THE UNIVERSITY *of* EDINBURGH

Determining the role of follicular dendritic cells in TSE agent neuroinvasion

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Declaration

I declare that the work presented in this thesis is my own, except where stated. All experiments were designed by myself, in collaboration with my supervisors Dr Neil Mabbott and Professor John Hopkins. No part of this work has been, or will be submitted for any other degree, or professional qualification.

Laura McCulloch

March 2011

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Abbreviations

aa	**Amino acids**
ABC	**Avidin-biotin complex method**
AP	**Alkaline phosphatase**
BAC	**Bacterial artificial chromosome**
BCR	**B cell receptor**
bp	**Base pairs**
BSE	**Bovine spongiform encephalopathy**
CJD	**Creutzfeldt-Jakob disease**
CNS	**Central nervous system**
CR1	**Complement receptor 1**
CR2	**Complement receptor 2**
Cre	**Cre recombinase**
CWD	**Chronic wasting disease**
DCs	**Dendritic cells**
d	**Days**
d.p.i	**Days post injection**
FACS	**Fluorescence-assisted cell sorting**
FDC	**Follicular dendritic cell**
FFI	**Fatal familial insomnia**
FSE	**Feline spongiform encephalopathy**
GSS	**Gerstmann Sträussler-Scheinker disease**
h	**Hours**
HRP	**Horseradish peroxidase**

ic	**Intracerebral**
ICAM-1	**Intracellular adhesion molecule-1**
Ig	**Immunoglobulin**
ILF	**Isolated lymphoid follicle**
ILN	**Inguinal lymph node**
ip	**Intraperitoneal**
Ig	**Immunoglobulin**
Kbp	**Kilobase pairs**
KDa	**KiloDaltons**
LoxP	**Locus of crossover P**
LT	**Lymphotoxin**
LtβR-Ig	**Lymphotoxin β receptor-human immunoglobulin fusion protein**
M	**Molar**
MAb	**Monoclonal antibody**
MBM	**Mammalian meat and bone meal**
MLN	**Mesenteric lymph node**
MZ	**Marginal zone**
ORF	**Open reading frame**
PAb	**Polyclonal antibody**
PAP	**Peroxidase-anti-peroxidase**
PBS	**Phosphate buffered saline**
PET	**Paraffin-embedded tissue**
PK	**Proteinase K**
Prnp	**Murine PrP gene**
PRNP	**Human PrP gene**

PrP	**Prion protein**
PrP^{c}	**Normal form of the host PrP protein**
PrP^{d}	**Disease-associated PrP protein**
PrP^{Sc}	**Scrapie-specific form of the host PrP protein**
sCJD	**Sporadic CJD**
SCID	**Severely combined immunodeficient**
SCS	**Sub-capsular sinus**
SRM	**Specified risk material**
TBMs	**Tingible body macrophages**
TH	**Tyrosine hydroxylase**
TNF	**Tumour necrosis factor**
TSE	**Transmissible spongiform encephalopathy**
VCAM-1	**Vascular cellular adhesion molecule-1**
vCJD	**Variant CJD**
WT	**Wild type**

Abstract

Transmissible spongiform encephalopathies (TSEs), such as scrapie and variant Creutzfeldt-Jakob disease are infectious, fatal, neurodegenerative diseases. Following peripheral infection TSE agents usually accumulate in lymhoid tissues before spreading to the central nervous system. In mice, follicular dendritic cells (FDCs) expressing the host prion protein (PrP^C) are essential for scrapie agent accumulation in lymphoid tissues. The accumulation of the scrapie agent on FDCs is critical for the efficient spread of infection to the brain. However, it is unknown whether FDCs themselves actively replicate the scrapie agent, or simply accumulate it following production by other cells types such as neurones, lymphocytes or other stromal cell populations. To definitively address this issue a transgenic mouse model was created in which PrP^C is switched on or off exclusively on FDCs.

Expression of cre-recombinase (Cre) under the action of cell-specific gene promoters can be used to induce or delete the expression of a target gene in specific cell populations. In this model, Cre expression is driven by the complement receptor type 2 gene (*Cr2*/CD21) which is expressed by FDCs and mature B lymphocytes. Characterisation of the *CD21-cre* mouse line was achieved by crossing with a *ROSA26* reporter strain. The *CD21-cre* mouse line was subsequently crossed with floxed-PrP mouse lines to produce compound transgenic mouse lines in which PrP^C expression was switched on or off, only in FDCs. Cre expression by B lymphocytes was eliminated by γ-irradiation and grafting recipient mice with Cre-deficient bone marrow. Immunohistochemical analysis confirmed the expression PrP^C had been switched on or off exclusively on FDCs. Subsequently, the mice were challenged with scrapie by intra-peritoneal injection to determine the precise role of FDCs in the accumulation of scrapie in lymphoid tissues.

Switching off PrP^C expression exclusively on FDCs prevented the accumulation of TSE agent specific disease-associated PrP^{Sc} in the spleen after i.p inoculation. Conversely, in mice in which PrP^C was expressed only on FDC, successful replication of the agent occurred on the FDC network in the spleen. Taken together, these data show PrP^C-expressing FDCs alone are sufficient to support the accumulation of the scrapie agent within lymphoid tissues. Furthermore, these data suggest FDC replicate the TSE agent and do not simply accumulate it following synthesis by other cell types.

CHAPTER 1

Introduction

1. Introduction

1.1 The transmissible spongiform encephalopathies (TSEs)

1.1.1 TSE diseases

The Transmissible spongiform encephalopathies (TSEs) are fatal diseases of the central nervous system (CNS) which affect various mammalian species, including humans (Table 1.1). These include Creutzfeldt-Jakob disease (CJD) and kuru in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in cattle. TSE diseases have a varied aetiology and can be sporadic, familial or acquired. These diseases are characterised by typical pathology in the CNS including spongiform change or vacuolation (Dickinson, Meikle et al. 1968), activation of glial cells, and abnormal accumulation of a host protein, in the brains of TSE-affected animals (Bolton, McKinley et al. 1982; Bruce, McBride et al. 1989). TSE diseases are difficult to study as various factors can impact on pathogenesis including host genotype (Dickinson, Meikle et al. 1968; Bruce, McConnell et al. 1991), route of inoculation (Eklund, Kennedy et al. 1967; Kimberlin and Walker 1979) and the strain of TSE used (Bruce and Fraser 1991). Additionally, in experimental studies the pathogenesis of TSE disease can exceed the lifespan of the infected animal which can complicate interpretation of incubation period data (Dickinson, Fraser et al. 1975). These barriers contribute to the many unknowns still present in TSE research including that the nature of the TSE agent itself, which is still as of yet not fully understood.

1.1.2 Scrapie

Scrapie is the earliest known TSE and affects sheep and goats and most of the knowledge we have of TSE disease has come from experimental transmission of scrapie into mice. In sheep, the disease is characterised by scratching and nibbling at skin leading to severe wool loss and skin damage, unco -ordination and recumbancy and results in severe weight loss and inevitably death. Scrapie is a centuries old disease, with the first traceable mention of the disease in a German publication from 1750 (Leopoldt 1750; Brown and Bradley 1998; Schneider, Fangerau et al. 2008a). Scrapie has so far been found in twelve EU member states including Britain, France and Germany, 3 candidate member states and Canada, US, Brazil and Japan. However two of the largest sheep producing countries of the world- Australia and New Zealand- remain scrapie-free.

Scrapie has been known to be infectious since its earliest traceable publication however the mechanisms of transmission and the nature of the infectious agent are still not fully understood to this day (Leopoldt 1750; Brown and Bradley 1998; Schneider, Fangerau et al. 2008a). The first definitive experiments to show scrapie transmission were carried out by Cuillé and Chelle in 1936. Animals were observed for 18 months to ensure they were scrapie-free before intraocular, subcutaneous, epidural or intracranial (ic) inoculation with brain and spinal cord homogenates from affected animals. They successfully transmitted scrapie to some, but not all of the animals inoculated, with incubation times between one and two years depending on the route of inoculation (Cuillé and Chelle 1936; Cuillé and Chelle 1938; Brown and Bradley 1998; Schneider, Fangerau et al. 2008a). Some infected homogenates were

passed through a bacterial exclusion filter prior to inoculation. These innocula successfully transmitted disease showing that the agent was not microbial (Cuillé and Chelle 1938; Brown and Bradley 1998; Schneider, Fangerau et al. 2008a). These experiments demonstrated for the first time that scrapie was transmissible by various routes, the disease has a long incubation period and showed differences in susceptibility between sheep. Transmission between species was also achieved by experimental inoculation of goats with sheep brain and spinal cord homogenates (Cuillé and Chelle 1939; Brown and Bradley 1998; Schneider, Fangerau et al. 2008a). Furthermore, accidental transmission of scrapie occurred via a new louping-ill vaccine prepared from brain, spinal cord and spleen homogenates of virus- infected sheep, attenuated with 0.35% formalin. This accidental transmission demonstrated that the spleen was also a source of scrapie-infected material and that the scrapie agent was resistant to treatment with formalin (Gordon 1946). The route of transmission of natural scrapie is still not fully understood. However it is known that disease can occur via both horizontal transmission, i.e. from sheep to sheep (Brotherston, Renwick et al. 1968), and lateral transmission, i.e. from ewe to lamb (Foster, Hunter et al. 1996; Foster, McKenzie et al. 2006).

Resistance to scrapie was known to be hereditary by the end of the 18th (Comber 1772) and beginning of the 19th (Thaer 1821; von Richthofen 1827; Schneider, Fangerau et al. 2008a) centuries, however effective genetic breeding selection methods in sheep were only applied recently by the European Commission when the genetics of scrapie were understood more fully (regulation 2001; regulation 2004; regulation 2007). In the 1960's, studies by Dickinson *et al* looking at ME7 scrapie incubation times in inbred mouse strains led to the discovery of the involvement of

the scrapie incubation period or "*Sinc*" gene in scrapie pathogenesis (Dickinson and Mackay 1964; Dickinson, Meikle et al. 1968). This group were also carrying out studies using the scrapie isolate SSBP/1 in sheep and concluded that the resistance of some sheep to SSBP/1 scrapie must be due to a single gene with a fully dominant allele for susceptibility (Dickinson, Stamp et al. 1968). In the 1980's, a 33-35 KDa protein, named the prion protein or PrP, was discovered to be the main component of scrapie-associated fibrils (SAF; (Oesch, Westaway et al. 1985; Hope, Morton et al. 1986). Subsequently, it was discovered that the *Sinc* gene encoded the prion protein (PrP) and confirmed the involvement of the normal cellular host prion protein (PrP^C) in TSE pathogenesis (Hunter, Hope et al. 1987). This discovery led to many studies of PrP sequence polymorphisms and their relevance to scrapie incubation time as well as much research into the role of PrP^C in health and disease.

1.1.3 Bovine spongiform encephalopathy

Bovine spongiform encephalopathy (BSE) is hypothesised to have emerged after cattle were fed on a diet containing mammalian meat and bone meal (MBM) derived from the rendered carcasses of scrapie-infected sheep (Wilesmith, Ryan et al. 1991; Anderson, Donnelly et al. 1996) . Cattle were not considered to be natural hosts of TSE disease however, the first outbreak of BSE was discovered in the 1980's (Wells, Scott et al. 1987). The development of BSE in cattle led to many subsequent cross species transmissions of TSE disease. Many animal feed products were contaminated with cattle-derived products containing infective material, which led to the development of feline spongiform encephalopathy (FSE) in domestic and exotic cats (Wells, Scott et al. 1987; Wyatt, Pearson et al. 1991), and novel TSE disease in other

exotic animals including nyala, kudu and oryx (Wells, Scott et al. 1987; Jeffrey and Wells 1988; Kirkwood, Wells et al. 1990) . This led to the mass culls of many infected cattle and the enforcement of new laws preventing the inclusion of MBM in cattle feed, the prevention of specified risk material (SRM) entering the food chain, the ban of mechanically recovered meat and meat from cattle over 30 months of age entering the food chain (regulation 2001; regulation 2004; regulation 2007).

1.1.4 Creutzfeldt-Jakob disease (CJD) and human TSEs

Human TSEs can be inherited, sporadic or iatrogenic. Genetic TSEs account for around 10% of human prion diseases. These include Gerstmann Sträussler-Scheinker disease (GSS), familial Creutzfeldt-Jakob disease (fCJD) and fatal familial insomnia (FFI) which are all associated with inherited mutations in the prion protein gene *PRNP* (Creutzfeldt 1920; Jakob 1921a; Jakob 1921b; Gerstmann, Straussler et al. 1936; Hsiao, Baker et al. 1989; Kretzschmar, Honold et al. 1991; Goldfarb, Petersen et al. 1992; Medori, Tritschler et al. 1992).

Sporadic CJD accounts for at least 85% of all human TSEs and occurs by as of yet unknown mechanisms, which lead to abnormal conformations of the normal host protein PrP^{C}. Possible hypotheses for the onset of sporadic TSE include acquired mutations of the *PRNP* gene or abnormal cellular metabolism of PrP^{C} which subsequently leads to conversion to the disease associated conformation (Westaway, DeArmond et al. 1994).

Iatrogenic, or peripherally acquired, human TSE diseases include kuru and variant CJD (vCJD). Kuru was discovered in 1957 and was transmitted during cannibalistic rituals of the natives of the Eastern Highlands of Papa-New Guniea (Zigas and Gadusek 1957). Kuru was restricted to this geographical location and was all but eradicated by raising awareness to prevent disease transmission. vCJD was first identified shortly after the BSE outbreak and most likely occurred via the consumption of BSE contaminated meat (Will, Ironside et al. 1996; Bruce, Will et al. 1997). As of the 9th March 2011, 171 deaths from vCJD have been reported in the U.K. with 4 patients currently living with probable vCJD (http//:www.cjd.ed.ac.uk/figures.htm). Furthermore, the more recent discovery of transmission of vCJD via contaminated blood products, along with the lack of a reliable *ante-mortem* diagnostic test or cure, has determined that these peripherally acquired TSEs remain a current public health issue (Llewelyn, Hewitt et al. 2004; Peden, Head et al. 2004; Wroe, Pal et al. 2006).

1.2 The TSE agent and TSE pathogenesis

1.2.1 The nature of the TSE agent

The exact nature of the TSE agent is yet to be determined and has been a source of controversy and debate. TSE diseases were originally thought to be caused by a virus due to the transmission of disease and the occurrence of multiple strains of each TSE. Due to their long incubation periods TSEs were considered to be a slow-virus, reproducing much more slowly than typical virus strains. However, a TSE-associated virus could not be detected by electron microscopy. Supporters of the virus

hypothesis suggested it may be extremely small, similar to some very small plant viruses such as Tobacco mosaic virus (Diringer, Gelderblom et al. 1983; Van Everbroeck, Pals et al. 2002).

This belief developed into the virino hypothesis, which stated that a very small nucleic acid, or some other information encoding molecule, is a component of the TSE agent (Dickinson and Outram 1988; Farquhar, Somerville et al. 1998). In support of this theory, a recently published paper claims to have found a 25 nm virion as the cause of TSE diseases (Manuelidis 2007) with the presence of tubovesicular structures found in TSE-affected brains subsequently associated with the presence of this virion (Liberski, Sikorska et al. 2008). As of yet, TSE disease specific nucleic acids have not been identified (Alper, Haig et al. 1966; Brown, Rohwer et al. 1986; Taylor 1993; Somerville, Birkett et al. 1997). However the existence of defined strains of TSE agents which are reproducible over multiple passages in mice has yet to be fully explained (Bruce and Fraser 1991; Bruce 1993).

In 1982, Stanley Prusiner published a paper stating that the TSE agent that caused scrapie contained no genetic material and consisted entirely of protein (Prusiner 1982). This conclusion was reached as the scrapie agent appeared to be resistant to inactivation by treatments that destroy nucleic acids such as ultra-violet light, ionising radiation, dry heat and nucleases (Alper, Haig et al. 1966; Prusiner 1982; Fraser 1987; Taylor 1993; Somerville, Birkett et al. 1997; Taylor, Fernie et al. 1998). Instead, proteinases, which destroy proteins, could either abolish or reduce infectious properties of scrapie (Prusiner 1982). Prusiner suggested that the infectious agent was the proteinaceous infectious particle, or prion, a novel type of protein-only pathogen

that lacks nucleic acid (Prusiner 1982). Fractions of scrapie-infected hamster brain were purified leading to the identification of a protein, termed PrP (Prion Protein), that was directly proportional to the titre of infectivity and was resistant to digestion by proteinase K (PK; (Bolton 1982; McKinley, Bolton et al. 1983). By testing for mRNA to PrP, it was found that PrP was a normal host protein (PrP^{C}; (Chesebro, Race et al. 1985) and that an abnormal form of this protein, PrP^{Sc} conferred disease (Meyer, McKinley et al. 1986). It was hypothesised that in prion pathogenesis, abnormal PrP^{Sc} can act as a template for converting normal PrP^{C} to the pathological variant (Rogers, Yehiely et al. 1993). Additionally, the recent *de novo* creation of infectious PrP^{Sc} *in vitro* seems to conclusively show that the TSE infectious agent is composed solely of protein (Legname, Baskakov et al. 2004; Barria, Mukherjee et al. 2009). The prion hypothesis can account for why no disease specific genetic material has thus far been identified and why viral inactivation treatments do not affect disease pathogenesis. The presence of TSE strains is a major factor in favour of the virino hypothesis, however it was proposed that TSE strain variation could be due to different conformations of PrP^{Sc} or differences in glycosylation of either the host PrP^{C} or the infecting PrP^{Sc} (Prusiner 1991; Ermonval, Mouillet-Richard et al. 2003). However, TSE infectivity has been shown in some models without the presence of PrP^{Sc} (Lasmézas, Deslys et al. 1997; Barron and Manson 2003; Barron, Campbell et al. 2007). Therefore, although there is compelling evidence for the prion hypothesis, clarification is required for some remaining phenomena of TSE pathogenesis.

1.2.2 The prion protein

Although the exact nature of the TSE agent is still controversial, PrP^{C} plays an important role in disease pathogenesis as $PrP^{-/-}$ mice are resistant to TSE disease

(Büeler, Aguzzi et al. 1993; Manson, Clarke et al. 1994b). PrP^C is a host encoded sialoglycoprotein expressed predominantly on neurons but also on many other cell types, including cells of the immune system (Kretzschmar, Prusiner et al. 1986; Caughey 1988). PrP^C is encoded by a single gene in rodents (*Prnp*), ruminants (*PrP or Prnp*) and man (*PRNP*) (Oesch, Westaway et al. 1985; Westaway and Prusiner 1986). The mouse gene has three exons and two introns, with exon 3 containing the entire protein coding region (Prusiner, Scott et al. 1998). The primary translation product in mouse has 254 amino acid (aa) residues, during posttranslational maturation the protein is processed to a length of approx. 210 aa (Hegde, Mastrianni et al. 1998a; Hegde, Voigt et al. 1998b) forming a flexible, unstructured N-terminal domain and a globular, highly structured C-terminal domain (Fig1.1).

After transcription and translation, the NH2-terminal signal peptide targets the protein to the endoplasmic recticulum (ER). This signal peptide is subsequently cleaved into the ER lumen (Hegde, Mastrianni et al. 1998a; Hegde, Voigt et al. 1998b). After the attachment of a glycosylphosphatidylinisotol (GPI) anchor, PrP^C protein is further modified through complex carbohydrates as it is transported along the ER-Golgi towards the plasma membrane at the cell surface. The PrP^C protein matures as it is transported along the ER-Golgi plasma membrane pathway from an immature form, which is sensitive to endoglycosidase H, to a mature form which is resistant to endoglycosidase H (Sarnataro, Campana et al. 2004). PrP^C becomes associated with lipid rafts in the ER and studies have shown that this is essential for the correct folding and glycosylation of PrP^C. Furthermore, association with rafts allows PrP^C to be exported from the ER to the Golgi. (Sarnataro, Campana et al. 2004; Campana, Sarnataro et al. 2005).

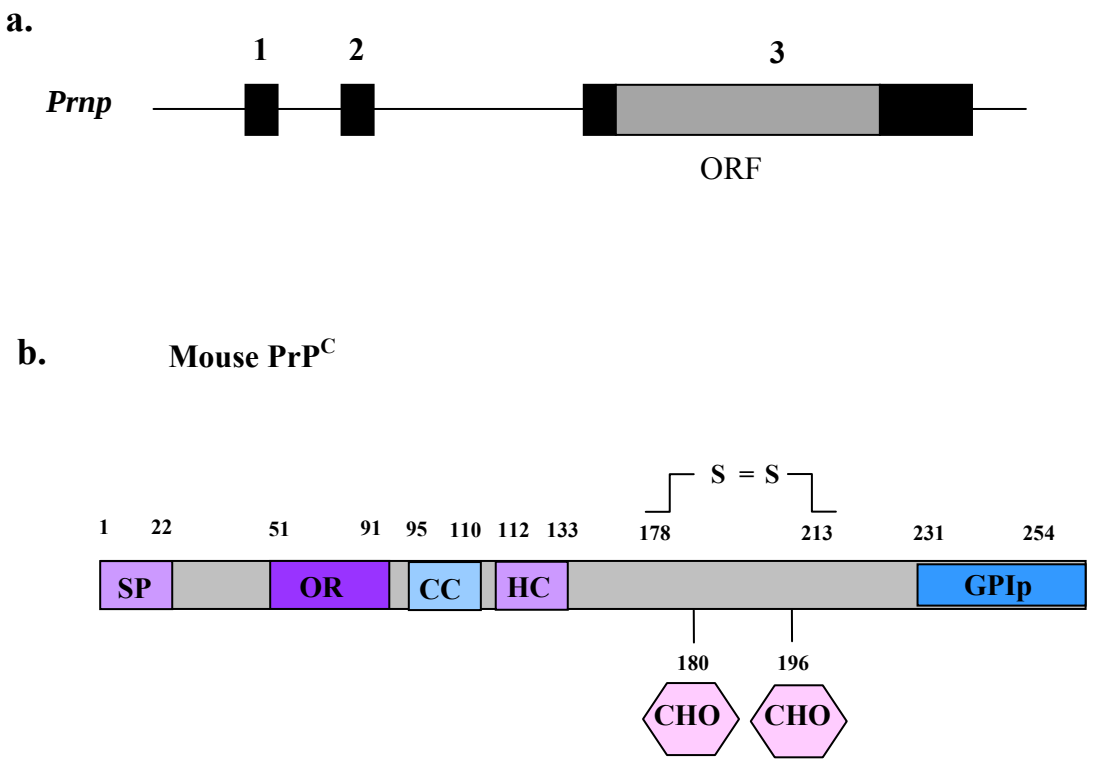


Figure 1.1 Structure of the mouse *Prnp* gene and PrP^C protein

a. Outline of the structure of the mouse *Prnp* gene. *Prnp* contains three exons and two introns. Exon 3 contains the open reading frame (ORF) which codes for the entire PrP^C protein.

b. Outline of the structure of PrP^C protein adapted from (Linden, Martins et al. 2008). The protein consists of a number of domains with specific functional attributes. It contains and N-terminal signalling peptide (**SP**) from aa 1-22 which targets the protein to the endoplasmic reticulum. The octapeptide repeat region (**OR**) is between aa 51-91 and is thought to have a role in copper binding (Hornshaw, McDermott et al. 1995; Jackson, Murray et al. 2001; Cerpa, Varela-Nallar et al. 2005). PrP^C contains both a charged cluster (**CC**) and a hydrophobic core (**HC**). PrP^C has two asparginine residues which are modified by N-linked glycans (**CHO**) at positions 180 and 196 and and this results in un-, mono- and di-glycosylated isoforms (Stimson, Hope et al. 1999). PrP^C also has a glycosyl phosphatidylinositol (GPI) anchor signalling peptide which allows the addition of a GPI anchor within the ER (Stahl, Borchelt et al. 1987).

PrP^C has a short half-life and is recycled via endosomes or degraded in lysosomes. As part of normal processing, PrP^C can be proteolytically cleaved into to fragments, with the N-terminal fragment secreted and the C-terminal fragment membrane-bound.

Expression levels of *Prnp* from microarray data from various mouse tissues and cell lines can be seen in Fig 1.2. The normal cellular function of PrP^C is uncertain and three distinct lines of $PrP^{-/-}$ mice show normal development and have no overt neurological phenotype (Bueler, Fischer et al. 1992; Manson, Clarke et al. 1994a; Prusiner, Scott et al. 1998; Lasmézas 2003). This suggests that either PrP^C is not an essential protein or that the genetic loss of PrP^C expression can be compensated for by other factors. Some proposed functions of PrP^C include the maintenance of circadian rhythm (Tobler, Gaus et al. 1996), synaptic transmission (Collinge, Whittington et al. 1994; Colling, Collinge et al. 1996; Mallucci, Ratte et al. 2002) anxiety modulation (Nico, de-Paris et al. 2005), cognition (Coitinho, Roesler et al. 2003) and seizure thresholds (Walz, Amaral et al. 1999) as small changes in these functions have been observed in the $PrP^{-/-}$ lines.

PrP^C has the ability to bind copper molecules through its sequence of octapeptide repeats and has therefore also been implicated in copper metabolism (Hornshaw, McDermott et al. 1995; Jackson, Murray et al. 2001; Lasmézas 2003; Cerpa, Varela-Nallar et al. 2005). More recent work claims that binding of copper ions may promote protein aggregation and consequently may be involved in pathogenesis (Shiraishi 2006). Additionally, PrP^C has been named as a signal transduction protein (Mouillet-Richard, Ermonval et al. 2000; Spielhaupter and Schätzl 2001), and has been suggested to have roles in both pro-apoptotic signalling via an associated

increase in caspase 3 activity (Paitel, Alves da Costa et al. 2002) and anti-apoptotic via binding to the anti-apoptotic molecule Bcl-2 (Kurschner and Morgan 1995; Kuwahara, Takeuchi et al. 1999; Bounhar, Zhang et al. 2001; Chiarini, Freitas et al. 2002; Paitel, Alves da Costa et al. 2002). The hypothesised functions of PrP^C are varied and as of yet it has proved difficult to distinguish its exact role within the CNS.

PrP^C is also expressed on many cells of the immune system including B and T lymphocytes, natural killer cells, platelets, monocytes, dendritic cells, mast cells and FDCs (Cashman, Loertscher et al. 1990; Barclay, Hope et al. 1999; Brown, Stewart et al. 1999; Holada and Vostal 2000; Li, Liu et al. 2001; Thielen, Antoine et al. 2001; Haddon, Hughes et al. 2009). Many of the putative functions of PrP^C in the CNS would not be required in cells of the immune system, however animal and human models have reported up- and down-regulation of PrP^C in certain immune conditions and differences in immune cell function when PrP is ablated. Maturation of DCs and monocytes has been reported to up-regulate PrP^C expression (Dürig, Giese et al. 2000; Burthem, Urban et al. 2001; Ballerini, Gourdain et al. 2006), whereas down-regulation has been reported upon activation of B and T lymphocytes in mice (Kubosaki, Yusa et al. 2001; Liu, Li et al. 2001). PrP^C is up-regulated in some functionally differentiated lymphocytes including a population of regulatory T lymphocytes (Huehn, Siegmund et al. 2004) and in memory CD8 T lymphocytes (Li, Liu et al. 2001; Goldrath, Luckey et al. 2004). PrP^C has also been linked to fixation of complement component within the immune system (Mitchell, Kirby et al. 2007). Furthermore, PrP^C is released in response to mast cell-mediated allergic inflammation (Haddon, Hughes et al. 2009). PrP^C appears to have an active functional role within cells of the immune system, however PrP^C deficiency has no impact on expression

levels of both MHC Class I and II, maturation of DCs, and numbers of $CD4^+$ and $CD8^+$ T lymphocytes and B lymphocytes are no different to that seen in WT counterparts (Bueler, Fischer et al. 1992; Kubosaki, Yusa et al. 2001; Ballerini, Gourdain et al. 2006; Zhang, Steele et al. 2006). Recent studies have shown that PrP^C deficient T lymphocytes are more susceptible to oxidative stress (Aude-Garcia, Villiers et al. 2011) which is in agreement with previous publications demonstrating a neuroprotective role against oxidative stress for PrP^C expression by neurones (Mitteregger, Vosko et al. 2007). This suggests PrP^C expression may have protective role in cells of both the CNS and immune system.

1.2.3 Distinguishing PrP^C from PrP^{Sc}

In TSE diseases, the conformational change of PrP^C to PrP^{Sc} has been shown to be a post-translational event, which involves changes in the content of α-helices and β-sheets (Rogers, Yehiely et al. 1993). Normal PrP^C has a structure consisting of around 40% α-helix and around 3% β-sheet. However, after its conformational change, PrP^{Sc} contains only 30% α-helix and around 45% β-sheet (Pan, Baldwin et al. 1993; Rogers, Yehiely et al. 1993). This increase in β-sheet content of PrP appears to confer some of the disease causing properties of PrP^{Sc}. For example, increased β-sheet content has been linked to protease resistance, insolubility in detergent and a propensity of the protein to aggregate (Meyer, McKinley et al. 1986; Somerville, Merz et al. 1986; Caughey, Dong et al. 1991) .

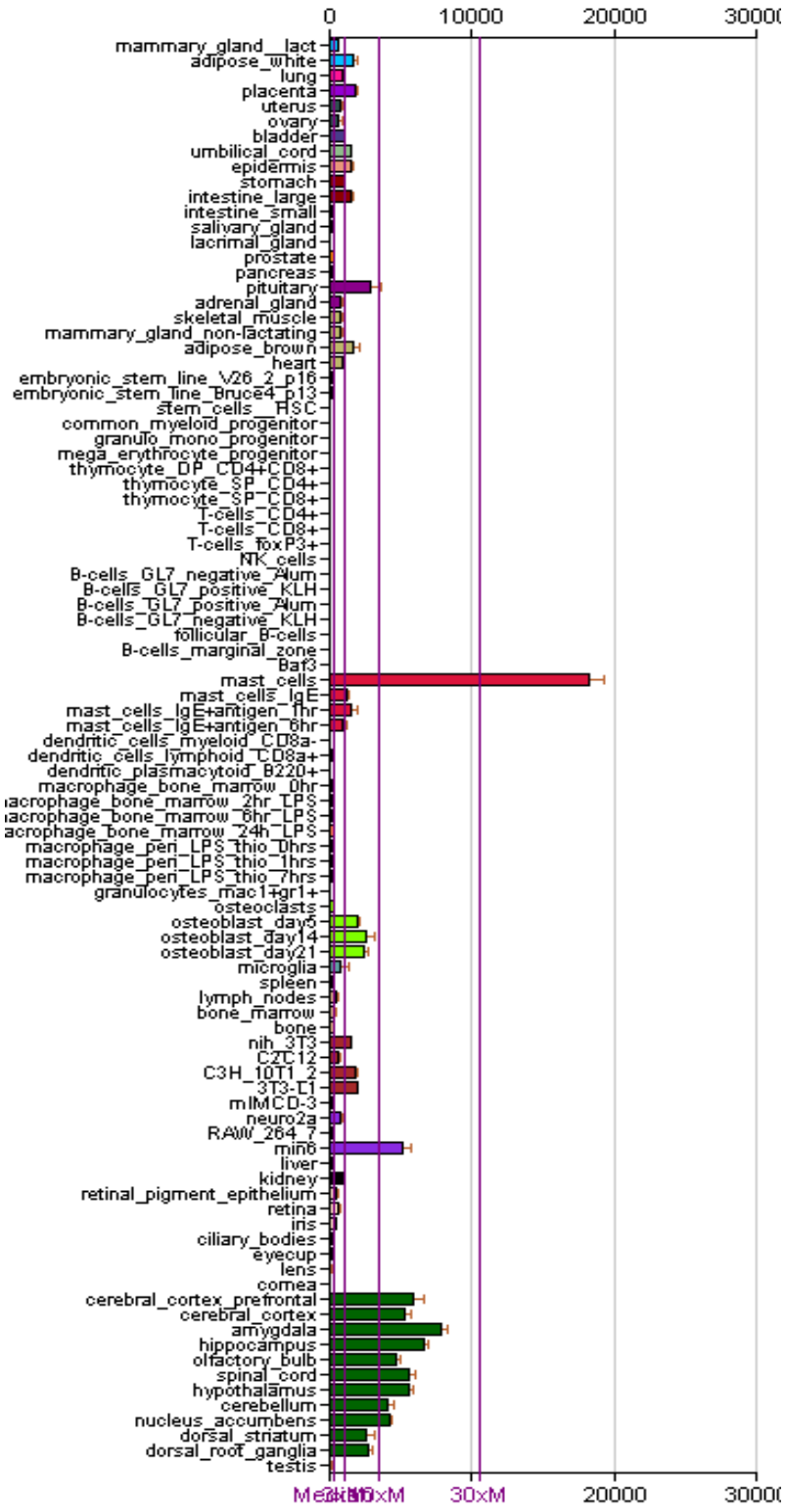


Figure 1.2 BioGPS expression profiles of *Prnp* in the mouse
Expression profiles of *Prnp* from BioGPS database (http://biogps.gnf.org), which stores microarray data of steady state mRNA expression in various murine tissues and cell lines. In agreement with published data, *Prnp* is ubiquitously expressed at low levels, however higher levels of expression are found within the CNS.

This latter characteristic is seen as fibrils of PrP^{Sc} forming in the brains of diseased animals (Meyer, McKinley et al. 1986; Pan, Baldwin et al. 1993; Prusiner, Scott et al. 1998). The partial proteinase resistance of PrP^{Sc} is thought to be conferred by a 27-30 KDa core which remains after treatment with proteinase (Bolton, Meyer et al. 1985). PrP^{Sc} deposition has been shown in numerous studies to be inseparable from infectivity (Bolton 1982; McKinley, Bolton et al. 1983) although this is not true for all TSEs as seen in studies using the P102L strain of GSS (Barron and Manson 2003; Barron, Campbell et al. 2007; Piccardo, Manson et al. 2007). Nevertheless, the deposition of PrP^{Sc} is widely accepted as a marker of infectivity.

Current antibodies available to detect PrP cannot distinguish between the cellular form, PrP^{C}, and the disease-associated form PrP^{Sc}. New antibodies are being developed which bind exclusively to PrP^{Sc} but are yet to be shown to be reliable for research application (Korth, Stierli et al. 1997; Paramithiotis, Pinard et al. 2003; Moroncini, Kanu et al. 2004; Jones, Wight et al. 2009; Petsch, Muller-Schiffmann et al. 2011). However, the biochemical properties of PrP^{Sc}, such as protease resistance and in solubility in detergents, allow PrP^{Sc} to be distinguished from PrP^{C} experimentally. For the histological detection of PrP^{Sc}, tissue sections can be fixed to nitrocellulose membrane and treated with proteinase K (PK) in the PET blot method (see section 2.4.3). PK denatures native cellular PrP^{C} leaving the PK-resistant PrP^{Sc} core intact (Schulz-Schaeffer, Tschoke et al. 2000). All PrP subsequently detected by immunolabelling can be confirmed to be PrP^{Sc}. PK digestion can also be used on tissue homogenates before immunoblotting. PK treatment of uninfected samples will result in no protein bands being detectable on a blot. Whereas, PK treatment of

infected samples results in the three PrP bands representing unglycosylated, monoglycosylated and diglycosylated isoforms of PrP (Oesch, Westaway et al. 1985).

1.2.4 Characteristics of TSE pathogenesis in the central nervous system and TSE strains

TSE infections are difficult to diagnose as some clinical symptoms are similar to other neurological diseases. The only definitive diagnosis can be made post-mortem via analysis of the brain. A TSE-infected brain shows characteristic neuropathology. As mentioned previously, PrP^{Sc} deposition can be used as a marker of infectivity and is seen at high levels in a brain at end-stage disease. In addition, there will be spongiform change, which is vacuolation in the brain tissue (Mikol 1999; Van Everbroeck, Pals et al. 2002). The areas in which this vacuolation occurs vary between TSEs but each member of the TSE family shows a distinct lesion profile upon transmission to experimental mice, which can be used to determine the type of TSE infection.(Fraser and Dickinson 1973). The final neuropathological characteristics are gliosis, which can occur due to the hypertrophy and/ or hyperplasia of the glial cells in the brain, and neuronal loss (Mikol 1999). Astrocytes are glial cells that provide trophic, metabolic and structural support to neurones. They additionally have roles in neuronal-glial communication, synaptic signalling, regulation of blood flow and can influence neuronal precursors or stem cells within the adult CNS (Seifert, Schilling et al. 2006). Astrocytosis is a common feature of many CNS pathologies, including TSE disease, and is thought to be a neuroprotective response to CNS injury (Liedtke, Edelmann et al. 1996). However, their exact involvement in the pathology/ protection of the CNS in TSE disease is currently

uncertain. Astrocytosis of TSE infected brain is commonly detected via glial fibrillary acid protein (GFAP), an intermediate filament (IF) superfamily member expressed by astrocytes (Schiffer, Giordana et al. 1986). Increased numbers of activated microglia are also seen within TSE-affected brains. Microglia are considered the macrophages of the brain are considered the macrophages of the brain and also respond to many CNS pathological conditions. Their transformation from the resting state to an activated state involves enlargement of cells and changes in morphology, up regulation of cell surface molecules and proteins, and increases in the numbers of membrane ruffles and projections involved in phagocytosis (Block, Zecca et al. 2007). Microglia are detected via the expression of ionised calcium-binding adaptor molecule-1 (Iba-1), which is constitutively and specifically expressed by microglia and macrophages (Ohsawa, Imai et al. 2004; Ladeby, Wirenfeldt et al. 2005). Detection of activated astrocytes and microglia using the previously described markers, in addition to spongiform change and deposition of the disease associated prion protein, provide useful markers to allow post-mortem diagnosis of TSE-affected brains.

Different TSE strains can be distinguished by their neuropathological characteristics and disease incubation periods in lines of inbred mice. Serially passaged strains of scrapie injected directly into the CNS via ic injection result in highly reproducible disease incubation times with all animals succumbing to clinical disease within a matter of days of each other and can be used to distinguish scrapie strains (Dickinson, Meikle et al. 1968; Dickinson and Meikle 1971). Additionally, the pattern of neuropathology in specific scoring areas of the brain can be used to create a lesion profile. Animals from the same inbred strain infected with the same strain of scrapie

give a characteristic distinctive lesion profile (Bruce, McBride et al. 1989). These methods of neuropathological characterisation can be used to distinguish TSE strains in inbred mice. Indeed, these methods were used to confirm that vCJD in humans was the same strain of TSE as that which causes BSE in cattle (Bruce, Will et al. 1997).

An additional method of characterising TSE strains is via the intensity and migration of PrP glycoform bands after immunoblotting. TSE strains show differences in the ratios of unglycosylated, monoglycosylated and diglycosylated isoforms of PrP^{Sc}, which migrate at different speeds using gel electrophoresis (Parchi, Castellani et al. 1995; Collinge, Sidle et al. 1996; Somerville, Chong et al. 1997; Somerville 1999). These ratios are characteristic to different strains and this method is used regularly to distinguish scrapie from atypical scrapie (Baron, Biacabe et al. 2007).

The TSE agent strain used in this thesis is the ME7 scrapie strain. This was originally derived from the spleen of a Suffolk sheep with natural scrapie that was intra-gastrically inoculated into mice. The ME7 strain was isolated after serial passages through inbred mouse lines (Zlotnik and Rennie 1965). ME7 is thought to be a prevalent strain of natural sheep scrapie as it is frequently isolated from pooled natural scrapie samples (Bruce, Boyle et al. 2002). It has been used in many experiments within the Neuropathogenesis Division and therefore the incubation period and neuropathology are well characterised for various inbred mouse lines. The pathogenesis of the ME7 strain includes a stage of replication within the lymphoid tissues followed by centripedal spread to other lymphoid tissues, peripheral nerves and subsequent CNS disease. This strain provides a useful model for peripherally

acquired TSE diseases which also have a stage of lymphoreticular replication prior to neuroinvasion and CNS disease. This is similar mechanism of pathogenesis to many natural scrapie infections of sheep and vCJD in humans (van Keulen, Schreuder et al. 1996; Hill, Butterworth et al. 1999; Bruce, Brown et al. 2000; Bruce, McConnell et al. 2001; Joiner, Linehan et al. 2002). For these reasons, ME7 provides a useful tool to model these peripherally-acquired TSE diseases in the lymphoid tissues.

However, other TSE diseases show extra-neural pathology but with a different mechanisim of pathogenesis. Diseases such as sCJD initiate within the CNS followed by dissemination of the agent to peripheral nerves and extra-neural pathology (Kitamoto, Mohri et al. 1989; Head, Northcott et al. 2003; Zanusso, Ferrari et al. 2003; Head, Ritchie et al. 2004). This is known as centrifugal spread and the ME7 scrapie strain would therefore not be a relevant model for this mechanism of pathogenesis.

1.2.5 The host immune response to TSE disease

It is considered that TSE infection does not generate a specific host immune response and no inflammation is seen in an infected brain. On considering the prion hypothesis, this may not be surprising, as the infectious agent appears to be an abnormally-folded form of a normal host protein. There is no PrP^{Sc} specific antibody response (Clarke and Haig 1966; Marsh, Pan et al. 1970; Porter, Porter et al. 1973) and deficiencies in circulating antibody or the Fc-γRI have no effect on pathogenesis (Klein, Kaeser et al. 2001). It is probable that the immune system is tolerized to the infectious TSE agent via its close resemblance to the normal cellular form of PrP

(Klein, Kaeser et al. 2001). This would occur via deletion of PrP-specific lymphocytes in early development of the immune system repertoire as an immune response against such an agent could provoke an autoimmune response. Furthermore, there is also a lack of a notable cell-mediated immune response against the TSE agent or PrP^{Sc} however, this is not due to any immunosuppressive effects caused by the agent (Kingsbury, Smeltzer et al. 1981).

However, in some but not all TSE strains, the TSE agent appears to undergo a stage of replication in the lymphoid tissue before disease progression to the CNS. Early experiments infecting mice with scrapie demonstrated accumulation and replication of the TSE agent within the spleen before neuroinvasion (Fraser and Dickinson 1970; Clarke and Haig 1971). Most natural sheep scrapie strains (van Keulen, Schreuder et al. 1996) and vCJD (Hilton, Fathers et al. 1998; Hill, Butterworth et al. 1999; Bruce, McConnell et al. 2001) are thought to require this replication stage for disease propagation, demonstrating that this could be an important area to target for early intervention in these diseases. More recent studies have shown that TSE infection has subtle effects on the immune system suggesting there may be consequences in immune function and a subtle immune response in response to infection in peripheral lymphoid tissues. Changes have been reported in the morphology of infected follicular dendritic cells (Jeffrey, McGovern et al. 2000; McGovern, Brown et al. 2004; McGovern and Jeffrey 2007; McGovern, Mabbott et al. 2009a). FDCs are cells found in the B lymphocyte follicles of peripheral lymphoid tissue which are discussed in more detail in Section 1.3.3. The FDC maturation cycle and the ability of infected mice to elicit an efficient germinal centre response are also reported to be affected in the spleens of scrapie-affected mice (Jeffrey, McGovern et al. 2000; McGovern, Brown et al. 2004; McGovern and Jeffrey 2007; McGovern, Mabbott et al. 2009a).

However it is important to remember that not all TSEs appear to require this stage of pathogenesis as is seen in BSE (Somerville, Birkett et al. 1997) and atypical or Nor98 scrapie (Buschmann, Biacabe et al. 2004; Vidal, Tortosa et al. 2008).

1.3 Lymphoreticular pathogenesis of the TSE agent

1.3.1 TSEs and the immune system

In many cases of TSE infection after peripheral exposure to the TSE agent, PrP^{Sc} deposition is seen in the gut-associated lymphoid tissue (GALT), including the Peyer's patches and mesenteric lymph nodes long before the deposition in the CNS (Kimberlin and Walker 1979; Andreoletti, Berthon et al. 2000). Early experiments using splenectomy of mice prior to scrapie inoculation were shown to prolong disease incubation period, suggesting that the spleen may be an important site for agent replication (Fraser and Dickinson 1970; Clarke and Haig 1971; Fraser 1978). However, splenectomy soon after inoculation had no significant effect on disease pathogenesis (Fraser and Dickinson 1970). Therefore, it was proposed that a functionally insignificant amount of the TSE agent remains in the spleen, while the majority replicates in other tissues (Fraser and Dickinson 1970).

Immunodeficient mice have also been used to demonstrate the necessity of lymphoreticular replication of certain TSE strains. SCID (Severe- Combined Immunodeficient) mice have no B or T lymphocytes or FDCs and are resistant to scrapie infection (Fraser, Brown et al. 1996). However, these cell types can be repopulated by grafting with wild type (WT) bone marrow, and doing so restores

susceptibility to disease (Fraser, Brown et al. 1996). These early experiments, amongst others, gave the first evidence that the immune system could have an important role in early disease pathogenesis.

1.3.2 The role of lymphocytes in TSE pathogenesis

SCID mice with no B- or T-lymphocytes are refractory to scrapie infection (Fraser, Brown et al. 1996). Early experiments investigating a role for B and T lymphocytes in TSE agent replication used immunodeficient mice. Thymectomy of mice before or after scrapie inoculation had no effect on scrapie incubation time or neuroinvasion, suggesting T-lymphocytes were not involved in TSE pathogenesis (Fraser 1978). This was confirmed using T lymphocyte-deficient mice with genetic deletions of CD4, CD8, perforin, β2μ or TCR-α which again showed no differences in disease pathogenesis, confirming that T-lymphocytes have no role in TSE agent replication (Klein, Frigg et al. 1997).

To investigate a possible role for B lymphocytes β2μ deficient mice with no B lymphocytes were inoculated with scrapie. These mice did not accumulate PrP^{Sc} or infectivity in their spleens and did not develop disease and B lymphocytes were suggested to replicate the TSE agent (Klein, Frigg et al. 1997). As PrP^{C} expression is essential for TSE transmission (Büeler, Aguzzi et al. 1993; Sakaguchi, Katamine et al. 1995). Expression of PrP^{C} exclusively by B lymphocytes was not sufficient to allow scrapie pathogenesis in the lymphoid tissue (Montrasio, Cozzio et al. 2001). Experiments using SCID mice and $RAG^{-/-}$ mice, which have no lymphocytes but express PrP^{C} equivalent to WT mice, were reconstituted with $Prnp^{+/+}$ or $Prnp^{-/-}$ bone

marrow before inoculation with scrapie. Expression of PrP^C by lymphocytes was shown to be irrelevant to TSE pathogenesis in this model (Klein, Frigg et al. 1998). This has since been confirmed with similar chimeric mouse models and with various strains of the TSE agent (Brown, Stewart et al. 1999; Loeuillet, Lemaire-Vielle et al. 2010). These experiments suggested that B lymphocyte products, rather than the cells themselves, were essential in TSE pathogenesis; or that pathogenesis may involve other cells dependent on B lymphocyte-derived signals for their development and/ or function. However, it is possible that B lymphocytes may assist in the spread of the TSE agent to other lymphoid organs. PrP^d was found associated with $CD21^+$ B lymphocytes in the blood of scrapie-affected sheep. Therefore it is possible that although B lymphocytes may not actively replicate the TSE agent, they may still have a role in pathogenesis via aiding the dissemination of the agent post-replication in other cells.

1.3.3 The role of FDCs in TSE pathogenesis

Initial studies examining TSE agent infectivity within the lymphoid tissues found that the stromal component of the spleen, which would have contained FDCs, contained more infectivity than the pulp (Clarke and Kimberlin 1984). FDCs are long-lived and mitotically inactive, making them resistant to ionising radiation which identifies them as a candidate for TSE agent replication as irradiation of mice has no effect on TSE pathogenesis (Fraser 1987). The expression of PrP^C has been shown to be essential for TSE pathogenesis and FDCs have relatively high amounts of PrP^C on their surface in uninfected mice (Brown, Stewart et al. 1999; Ritchie, Brown et al. 1999). Immunohistochemical analysis of TSE-infected lymphoid tissue in mice shows strong

immunolabelling of PrP^{Sc} associated with the FDCs, which can be used as a biochemical marker of TSE infectivity (McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Ritchie, Brown et al. 1999; Bruce, Brown et al. 2000). Furthermore, labelling of the disease-associated PrP^{d} is also found in FDCs of sheep with natural scrapie (van Keulen, Schreuder et al. 1996), deer and elk with chronic wasting disease (Sigurdson, Williams et al. 1999) and humans with vCJD (Hill, Zeidler et al. 1997). Electron microscopy of infected lymphoid tissue showed high accumulation of PrP^{Sc} on the FDC surface and around the dendrites (Jeffrey, McGovern et al. 2000).

Mice with genetic deficiencies in tumour necrosis factor (TNF)-α have a lack of terminally differentiated FDCs but do have functional and mature T and B lymphocytes (Pasparakis, Alexopoulou et al. 1996). $TNF\alpha^{-/-}$ mice are resistant to TSE infection after peripheral exposure (Mabbott, Williams et al. 2000a). The TNF-signalling pathway can be disrupted treatment with a TNF-R1 homologue that causes a temporary dedifferentiation of FDCs but no disruption to lymphocyte populations. These TNF-depleted mice have increased disease incubation times on infection with ME7 scrapie, suggesting a role for FDCs in the peripheral replication of the scrapie agent (Mabbott, McGovern et al. 2002). However, a few mice in this study did develop disease after the same incubation as the control mice raising the question of whether FDCs are the only cell involved in TSE agent replication (Mabbot 2002). TNF-α is also an important cytokine for macrophage activation, meaning that TNF depleted mice would show some deficiencies in this process. It is possible that macrophages are involved in taking up and degrading the infective agent early after inoculation. In this instance, disease may have occurred in some of the TNF depleted

mice due to the lack of early sequestering and degradation of the agent by macrophages (Mabbott, McGovern et al. 2002). It is also possible that the agent may be able to replicate in unactivated macrophages as the TNF-induced degradation processes, such as lysosome activation and superoxide formation, will not have been activated. This was demonstrated by Prinz *et al* who inoculated TNFR1$^{-/-}$ mice with Rocky Mountain Laboratory (RML) scrapie strain and noted prion protein accumulation associated with some macrophages in the absence of FDC networks (Prinz, Montrasio et al. 2002).

Signalling through the lymphotoxin (LT) β Receptor (LTβR) on FDCs is required for maintenance of a mature FDC network and blocking this signalling results in their dedifferentiation to an immature state. These mice still retain functional lymphocytes, macrophages and DCs (Mackay and Browning 1998). FDCs can be dedifferentiated temporarily using LTβR-Ig, which acts as a soluble decoy receptor, binding LTβ ligands and preventing their interaction with LTβR. The blockade of LTβR-signalling before inoculation with ME7 scrapie can significantly extend the survival time of the mouse and reduces disease susceptibility (Mabbott, Mackay et al. 2000b). However, some animals eventually succumbed to clinical disease, therefore it may be that infectivity can persist in some other cell type until the FDC network reforms and the agent can begin to replicate (Mabbott, Mackay et al. 2000b). LTβR signalling can also be disrupted completely using knockout mice and studies have looked at the differences between LTα and LTβ deficient mice. These mice have similar reductions in their FDC populations, but LTα knockout mice appear to be more susceptible to RML scrapie than LTβ knockouts (Oldstone, Race et al. 2002). This suggests the possible involvement of another, unidentified LTβ-dependent cell type

which may also have a role in lymphoid replication of the TSE agent. Alternatively, LTβ may have additional effects on FDCs, distinct from those shared with LTα, that as of yet are unknown.

The CXCR5 receptor on B lymphocytes responds to stimulation via CXCL13 secreted by FDCs and is important in organising the structure of the follicle. A deficiency in CXCR5 disrupts the follicle structure, placing FDCs adjacent to the peripheral nerve endings found near the central arterioles of the spleen (Voigt 2000). These mice were inoculated with RML scrapie and the speed of neuroinvasion was found to be greatly increased when compared to the wild type (Prinz, Heikenwalder et al. 2003). This suggested that FDCs replicate the TSE agent prior to neuroinvasion and the speed of neuroinvasion can be increased by manipulating distance between the FDCs and nerve endings. As a control, this was repeated in $CXCR5^{-/-}$ mice with their FDCs depleted. These mice did not develop scrapie, confirming that the hastened disease progression was due to the displacement of the FDCs (Prinz, Heikenwalder et al. 2003).

These studies provided strong evidence that FDCs are responsible for replicating the TSE agent in the lymphoid tissues. However, FDCs are specialised in taking up and retaining proteins on their surface, therefore, it is uncertain whether the FDCs are actively replicating the TSE agent or instead are accumulating agent after replication in other cells. This question was addressed using chimeric mice which have mismatches in PrP^{C} expression between their FDCs and their bone marrow-derived cells. SCID mice have no FDCs due to their lack of B cells but FDC populations of host genotype can be restored by grafting with donor bone marrow (Humphrey,

Grennan et al. 1984; Kapasi, Burton et al. 1993; Brown, Stewart et al. 1999). Inoculating these PrP^C chimeric mice with ME7 scrapie showed that scrapie pathogenesis was dependent on FDC expression of PrP^C, regardless of expression status of bone marrow-derived cells . This has been confirmed by recent studies which have also shown PrP^C-expressing bone marrow derived cells are not responsible for replicating the TSE agent (Loeuillet, Lemaire-Vielle et al. 2010). From this it appears that FDCs themselves actively replicate the TSE agent. However, non-bone marrow-derived cells such as neural, epithelial and stromal cells within the lymphoid tissue retained expression of PrP^C in this model. Therefore, this does not exclude the possibility that FDCs simply acquire PrP^{Sc} from other cell types rather than replicating the agent themselves. To definitively determine the role of FDCs in scrapie pathogenesis an FDC-specific model is required that allows the role of these cells to be assessed in isolation from all other cell types within the lymphoid tissue.

1.3.4 The role of macrophages in TSE pathogenesis

Macrophages were also investigated as a possible cellular host for TSE agent replication. Tingible body macrophages (TBMs) reside in the follicles of lymph nodes where their main role is the clearance of proteins and apoptotic cells, many of which are produced in the germinal centre reaction during an immune response (Swartzendruber and Congdon 1963). Electron microscopical analysis of infected lymphoid tissue found PrP^{Sc} within TBMs of scrapie-affected sheep and mice (Jeffrey, McGovern et al. 2000; Jeffrey, Martin et al. 2001; Ryder, Dexter et al. 2009) and was also present in TBMs of lymphoid tissues of vCJD patients (Hilton, Ghani et al. 2004). Additionally, *in vitro* incubation of the scrapie agent with peritoneal

macrophages led to an association of the agent with macrophages (Carp and Callaghan 1981) and incubation with synthetic PrP peptide 106-126 was found to slightly stimulate macrophages causing up-regulation of PrP^{C} and TNFα production (Zhou, Xu et al. 2008). However incubating ME7 scrapie with macrophages prior to inoculation, led to an increased incubation period in mice in comparison to non-macrophage incubated ME7 (Carp and Callaghan 1982). This indicated that some of the scrapie had been inactivated or degraded by the macrophages and therefore macrophages would not be an adequate host cell for agent replication. Additionally, PrP^{Sc} detected in macrophages was usually associated with lysosomes suggesting macrophages were degrading the scrapie agent (Jeffrey, Martin et al. 2003; McGovern and Jeffrey 2007; McGovern, Mabbott et al. 2009a). To further confirm these findings, depletion of splenic macrophages using clodronate just before or after inoculation with scrapie leads to decreased incubation period and accelerated disease progression. Immunolabelling of PrP^{Sc} within TBMs using a panel of antibodies that bind different epitopes of PrP determined that PrP^{Sc} found within TBMs was truncated with a loss of 23-90 aa from the N-terminus (Jeffrey, Martin et al. 2003; McGovern, Mabbott et al. 2009a). Evidence suggests that macrophages do take up the TSE agent but degrade and destroy it and therefore macrophages would not be an adequate host cell for replication to occur in.

1.3.5 The role of dendritic cells in TSE pathogenesis

Dendritic cells (DCs) are bone marrow-derived cells of a distinct origin and function to that of FDCs. They form the body's immune surveillance by patrolling the periphery and sampling antigen in their local environment, subsequently migrating to

the draining lymph node and presenting it to T lymphocytes. Therefore, DCs have also been investigated as a candidate cell for the uptake and replication of the TSE agent in the lymphoid tissues. *In vitro* studies have shown that rat DCs are able to take up and retain scrapie for up to 72 hours (Huang, Farquhar et al. 2002) and PrP fragment 106-126 has been reported to be chemoattractant to immature DCs (Kaneider, Kaser et al. 2003). DCs have been shown to take up scrapie agent from the gut and transport it to the lymph node (Huang, Farquhar et al. 2002). This was confirmed by recent studies which showed DC-like cells containing PrP^{Sc} are present in the villus lacteals, submucosal lymphatics and sinuses of mesenteric lymph nodes 1.5- 24 h post oral inoculation with scrapie, further suggesting that DCs transport the scrapie agent to the lymphoid tissue (Jeffrey, González et al. 2006). Selective depletion of $CD11c^{+}$ DCs prior to oral inoculation with scrapie resulted in prolonged incubation periods, further supporting a role for DCs in the uptake and transport of the TSE agent (Raymond, Aucouturier et al. 2007). However, in this model, some animals eventually succumbed to disease suggesting that the TSE agent is able to utilise other cell types. Evidence so far supports a role for DCs in the uptake of the TSE agent, which is thought to be aided by complement opsonisation of the TSE agent (Flores-Langarica, Sebti et al. 2009) and subsequent transportation to the lymphoid tissues. However ionising radiation, which would deplete all DCs, has no effect on scrapie pathogenesis (Fraser and Farquhar 1987; Fraser, Farquhar et al. 1989) and bone marrow chimeric models have shown that TSE agent pathogenesis is not dependent on PrP^{C} expressing bone marrow-derived cells (Klein, Frigg et al. 1998; Brown, Stewart et al. 1999; Loeuillet, Lemaire-Vielle et al. 2010). Therefore it appears that DCs do not replicate the TSE agent but may be involved in initial localisation of the agent to the lymphoid tissue after some routes of peripheral

exposure. DCs may also be involved in the dissemination of the TSE agent after replication on the FDC networks and subsequent neuroinvasion as $CD11c^+$ splenic DCs were shown to be able to transport the TSE agent to the CNS in mouse scrapie 139A (Aucouturier, Geissmann et al. 2001).

1.3.6 The role of complement in TSE pathogenesis

The complement system is a cascade of heat labile serum proteins that can interact with pathogens to mark them for destruction. Complement can initiate an immune response by inducing inflammation, recruiting phagocytic cells, opsonising pathogens and forming immune complexes for clearance and lysis of certain pathogens and cells. An additional function of complement is its importance in the binding of antigenic immune complexes to the FDC surface via its CD21 and CD35 complement receptors (van der Berg, Yoshida et al. 1995). It is known that C3 is essential for the follicular localisation of antigen in an immune response therefore, complement may also be important in the localisation of the TSE agent to the follicle via the binding of the TSE agent to the FDC network via its expression of complement receptors.

The importance of complement in scrapie pathogenesis was investigated by both genetic deletion of C3 (Klein, Kaeser et al. 2001) and temporary removal of plasma C3 using cobra venom factor, which inhibits antigen localisation to the follicle (Mabbot 2001). Both of these studies showed increased incubation time and delayed neuroinvasion with intra-peritoneal (ip) inoculation of RML and ME7 scrapie respectively. However, infectivity titres in the spleen were similar to WT control mice, suggesting that although C3 may be involved in localising the TSE agent to the

lymphoid tissue it may not be important in the subsequent replication and neuroinvasion of the agent. Deletions of complement components associated with the terminal pathway, such as C5, have no effect on TSE pathogenesis, suggesting that this complement pathway has no role in the pathogenesis of the TSE agent (Mabbott 2004). Furthermore, genetic deletions of complement components appear to have no effect on TSE-associated neuropathology, suggesting that neurodegeneration in TSE infection is not complement-mediated (Mabbott, Bruce et al. 2001)

Immune complexes, can also bind to FDCs via their Fc receptors, however depletion of these receptors had no effect on RML scrapie pathogenesis (Klein, Kaeser et al. 2001). Therefore the localisation of the TSE agent to the FDC is unlikely to be mediated via immunoglobulins. The C1q complement component binds antigen complexes and can mediate their uptake directly via its receptor, CD35, or by activating C4 (Klickstein, Barbashov et al. 1997). Recent studies have shown that PrP^{Sc} can directly bind C1q via covalent interactions and activate complement via the classical pathway (Mitchell, Kirby et al. 2007). In addition, it is thought that FDCs themselves can synthesise C1q aiding immune complex formation and localisation of antigen to the follicle (Schwaeble, Schafer et al. 1995). These studies suggest that complement components, especially C1q, have a role in the localisation of the TSE agent to the lymphoid tissue.

1.4 The origin and function of follicular dendritic cells

1.4.1 Function of the FDC network

Follicular dendritic cells (FDCs) are currently thought to be the cells in the lymphoid tissue responsible for replicating the TSE agent. Prior to initiation of an immune response, FDCs are found within the primary follicle of secondary lymphoid tissues where they form networks of long dendrites and maintain the structural integrity of the B lymphocyte follicle (Heinen, Bosseloir et al. 1995). After exposure to antigen, primary follicles form germinal centers which contain FDCs, B lymphocytes, helper $CD4^+$ T lymphocytes and TBMs. FDCs aid this process via the secretion of chemokines which result in the recruitment of these cells to form the germinal centre (Kosco, Pflugfelder et al. 1992; Kosco-Vilbois, Bonnefoy et al. 1997; Kosco-Vilbois 2003). The structure of the germinal centre ensures the FDCs have close contact with large numbers of B lymphocytes, which is essential for their function. FDCs are specialised to capture antigen in the form of immune complexes but are non-phagocytic and have no class II MHC expression (Gray and Skarvall 1988; Kosco, Pflugfelder et al. 1992; Maeda, Matsuda et al. 1995; van der Berg, Yoshida et al. 1995). Instead these immune complexes are retained on their surface for long periods of time and are thought to be important in B lymphocyte development, affinity maturation, antibody class switching and maintenance of B lymphocyte memory (Gray and Skarvall 1988; Gray, Kosco et al. 1991; Kosco, Pflugfelder et al. 1992; Heinen, Bosseloir et al. 1995). This occurs via positive selection of anti-apoptotic signals to B cells with highest antigen affinity. To allow immune complex capture, FDCs express complement receptors CR2 (CD21) and CR1 (CD35) and FcγRIIb on

their surface and may additionally have complement receptor 3 (CR3) and FcεRII (Qin, Wu et al. 2000; Balogh, Aydar et al. 2001). Fc receptors have high affinity for the Fc fragment of immunoglobulin molecules and allow FDCs to bind immune complexes of antigen opsonised by IgG. This is aided by the expression of the complement receptors. CD21 has a high affinity for the complement fragments C3dg and C3d, whereas CD35 binds the complement fragments C3b and C4b, allowing FDCs to bind antigens opsonised by these (Liu, Xu et al. 1997). Other possible surface markers found on FDCs include some usually found on B cells, including CD20, CD22, CD24 and CD45 however, this is difficult to determine by immunohistochemistry due to FDC close proximity to B lymphocytes *in vivo* (Heinen, Bosseloir et al. 1995; van der Berg, Yoshida et al. 1995). During the germinal centre response FDCs also up-regulate the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Kosco, Pflugfelder et al. 1992; Heinen, Bosseloir et al. 1995; Nielsen 2000; Videm and Albrigtsen 2008).

FDCs produce IL-6, which is important for the organisation and maintenance of the germinal centre reaction (Kosco-Vilbois, Bonnefoy et al. 1997; Kopf, Herren et al. 1998). This IL-6 is thought to amplify production of complement component C3 by local TBMs, which may subsequently increase the formation of immune complexes and attachment of these to the FDCs (Kopf, Herren et al. 1998). FDCs also produce the chemokine CXCL13, which attracts B cells to the follicle via their CXCR5 receptor (Kosco, Pflugfelder et al. 1992; Heinen, Bosseloir et al. 1995; Kosco-Vilbois, Bonnefoy et al. 1997).

FDCs have recently been discovered to be aid the uptake of apoptotic B cells in the germinal centre by tingible body macrophages (TBMs). FDC-M1 or monoclonal antibody (MAb) clone 4C11 is considered to be a useful marker for FDCs as its expression is restricted to FDCs and TBMs. However until recently the epitope recognised by this MAb was unknown (Kosco, Pflugfelder et al. 1992) . Kranich *et al* noted that milk fat globule epidermal growth factor 8 knockout (*Mfge8*$^{-/-}$) mice had no FDC-M1^{+} FDC networks in the lymphoid tissues although the FDCs were detectable by CD21/35 immunolabelling. They confirmed that MFGE-8 is the same antigen as the epitope recognised by MAb FDC-M1 and is secreted by the FDCs to opsonise apoptotic B cells in the germinal centre. This mediates the clearance of apoptotic B cells by TBMs and is essential for the prevention of autoimmune disorders (Hanayama, Tanaka et al. 2004; Kranich, Krautler et al. 2008). Secretion of MFGE-8 is possibly driven by signalling through the lymphotoxin β receptor (LTβR) as MFGE-8 levels are greatly reduced in LTβR$^{-/-}$ mice (Kranich, Krautler et al. 2008).

1.4.2 The origin and development of the FDC network

The origin of FDCs is controversial but the predominant hypothesis is that they are non-bone marrow-derived cells and instead are derived from stromal cell precursors. One reason for this opinion is that FDCs share many surface markers with fibroblasts and can form ectopically under chronic inflammatory conditions, for example in the inflamed synovium of rheumatoid arthritis patients (Heinen, Bosseloir et al. 1995; Maeda, Matsuda et al. 1995). Initial experiments showed that animals which were irradiated and reconstituted with bone marrow consistently had FDCs of host origin (Humphrey, Grennan et al. 1984). This was confirmed in later studies using SCID

mice which have no FDCs. Grafting these mice with donor bone marrow can indirectly restore FDC networks via B-lymphocyte-derived signals. Work by Kapasi *et al* showed that newly generated FDCs in SCID mice were consistently of host genotype and not that of donor bone marrow (Kapasi, Burton et al. 1993). This procedure was subsequently used to create mice with mismatches in PrP^C expression with the newly generated FDCs consistently of host PrP^C expression status and not that of donor bone marrow, further suggesting FDCs are not bone marrow-derived cells (Brown, Stewart et al. 1999). Another experiment used transplantations of human cord blood into RAG-$2^{-/-}$ mice, which also have no B or T lymphocytes or FDCs. This resulted in the generation of murine, and not human, FDCs even though animal was repopulated with human blood cells (Traggiai, Chicha et al. 2004). FDC-derived cell lines have been shown to express many surface proteins relating them to bone marrow stromal cell progenitors and the expression of α-smooth muscle actin on FDC cell lines specifically related them to myofibroblasts (Munoz-Fernandez, Blanco et al. 2006).

In contrast, some studies argue that the FDC precursor is derived from the bone marrow and not the stromal cell population (Mebius, van Tuijl et al. 1998). Various authors have suggested bone marrow-derived cells such as $CD5^+$ B-1B lymphocytes or antigen transporting cells such as DCs can act as inducer cells for the formation of the FDC networks (Szakal and Tew 1992; Wen, Shinton et al. 2005). Kapasi et al reconstituted SCID mice with donor bone marrow and found cells expressing donor antigens and the FDC marker FDC-M1 in the newly formed lymphoid tissues (Kapasi, Qin et al. 1998). However, FDCs are known to be able to acquire proteins on their surface that they themselves do not express, therefore it is possible that FDCs

acquired these antigens from donor B lymphocytes, rather than the FDC networks developing from donor bone marrow precursors (Gray, Kosco et al. 1991; Denzer, van Eijk et al. 2000).

A recent study by Murakami et al proposed to have found a cell type that induces the formation of FDC networks. $CD19^{-}CD11c^{-}CD35^{+}B220^{+}$ inducer cells were separated from splenocytes, and injected intradermally along with stromal like $CD45^{-}CD35^{+}$ cells to successfully induce the formation of lymphoid tissue-like structures. Furthermore, GFP-tagged inducer cells demonstrated that these inducer cells gain expression of the FDC marker FDC-M1 in the newly formed follicles. This was confirmed by adoptive transfer of GFP-tagged inducer cells into naïve C57BL/6J-Jcl mice resulting in GFP^{+} FDCs in the reticulum of the spleen (Murakami, Chen et al. 2007). The inducer cells were able to retain immune complexes on their surface both *in-vitro* and *in-vivo*, a major distinguishing feature of FDCs. These data strongly suggest a role for these $CD19^{-}CD11c^{-}CD35^{+}B220^{+}$ inducer cells in the formation of the FDC network. The development of the FDC network is also dependent on B cell-derived lymphotoxins (Chaplin and Fu 1998), especially the membrane-bound LT. $LT\alpha^{-/-}$ or $LT\beta^{-/-}$ mice, have defects in the formation of lymph nodes and no Peyer's patches (Chaplin and Fu 1998). $TNF^{-/-}$ mice also had a similar phenotype, suggesting this is also involved in FDC development (Chaplin and Fu 1998). It has since been confirmed that signalling through the p55 TNFR on FDC is essential for formation of the FDC network and maintenance of FDC function (Victoratos, Lagnel et al. 2006). Furthermore, these signals are not just required for initial FDC development, continual signalling via $LT\alpha1\beta2$ secreted from B cells is also required for

maintenance of the FDC network structure as blocking this signal using a soluble LTβ receptor causes the networks to dedifferentiate (Mackay and Browning 1998).

1.4.3 FDC-independent TSE agent replication

From the work mentioned previously, it seems certain that FDCs have a role in TSE pathogenesis, however some conflicting results have shown that they may not always be required. Some naturally occurring TSE diseases appear to be restricted to the CNS, with no PrP^{Sc} deposition or infectivity found in the extraneural tissues. For example, no extraneural disease was detected in sCJD patients (Hill, Butterworth et al. 1999) until a method of concentrating PrP^{Sc} to increase the sensitivity for western blot was used and PrP^{Sc} was subsequently detected in some of the samples tested (Glatzel, Abela et al. 2003). This is in contrast to vCJD where infectivity and PrP^{Sc} deposition have been reported in various tissues including the spleen and tonsils (Hill, Butterworth et al. 1999). Additionally, disease appears to be restricted to the CNS in cattle naturally infected with BSE (Somerville, Birkett et al. 1997). In contrast, PrP^{Sc} has been detected in the PP of the distal ileum in cattle experimentally infected with disease (Terry, Marsh et al. 2003) and experimental inoculation of BSE into sheep has also resulted in PrP^{Sc} deposition in the lymphoid tissues (Lemzi, Ronzon et al. 2006). Atypical forms of BSE and scrapie have recently been described and there also appears to be no involvement of the lymphoid tissues with these TSE strains (Buschmann, Biacabe et al. 2004; Benestad, Arsac et al. 2008). A case of atypical scrapie with a concurrent, non-related inflammation in the brain was recently described. This animal was detected as having atypical scrapie by active surveillance and also had virus induced, non-purulent encephalitis which had led to the formation

of ectopic lymphoid follicles in the brain tissue. Examination tissues from this animal showed that there was no association of PrP^{Sc} with any draining lymph nodes or ectopic follicles found in the brain tissue (Vidal, Tortosa et al. 2008). This lack of PrP^{Sc} accumulation on ectopic follicles in the brain further demonstrate the lack of lymphoreticular involvement of the atypical scrapie strain as inflammatory conditions in various organs which cause the formation of FDC-containing ectopic follicles, have previously shown to be a favourable environment for PrP^{Sc} accumulation (Heikenwalder, Zeller et al. 2005). Furthermore, PrP^{Sc} accumulation in inflamed excretory organs such as the kidney or mammary glands is thought to aid spread of scrapie through shedding of the TSE agent in urine or milk respectively (Heikenwalder, Zeller et al. 2005; Ligios, Sigurdson et al. 2005; Ligios, Cancedda et al. 2007; Konold, Moore et al. 2008). These studies suggest that there may be variations in TSE strain cellular targeting.

Recent evidence has suggested that the FDCs themselves might not be essential for PrP^{Sc} replication but that any stromal cell with some FDC-like functions could theoretically support prion replication. Heikenwalder *et al* created a model in which TSE agent replication occurred in complete Freunds adjuvant (CFA) -induced granulomas in the skin following ip inoculation. Analysis of the granuloma showed a complete lack of commonly used FDC markers and FDC-specific, *Mfge8* mRNA but relatively high levels of PrP^{C}. However, administration of the LTβR-Fc, which has been shown to dedifferentiate FDCs, reduced TSE agent accumulation in the granulomas (Mackay and Browning 1998; Heikenwalder, Kurrer et al. 2008). Furthermore, these stromal cells expressed high levels of PrP^{C} and organised lymphocytes within the granuloma into follicle-like structures. Analysis of this model

led to the conclusion that that scrapie replication was occurring on a radio-resistant fibroblastic cell which did not express any FDC-specific markers and that this occurred in a LTβ-dependent manner (Heikenwalder, Kurrer et al. 2008). This is in accordance with the finding that unaltered, mouse derived fibroblast cell lines can be infected with RML scrapie *in vitro* and are sufficient to sustain PrP^{Sc} replication (Vorberg, Raines et al. 2004). Muscle cell lines have also been shown to support prion replication with finding of PrP^{Sc} in the muscle tissue of a sporadic CJD patient confirming this (Kovacs, Lindeck-Pozza et al. 2004; Dlakic, Grigg et al. 2007). Therefore it may be possible that any stromal cell with an FDC-like function has the capacity to support TSE agent replication.

Natural and experimental strains of TSE disease show variation in pathogenesis thought to be due to genetic or strain properties, but there are also reported differences between groups using the same agent strain. For example, Klein *et al* inoculated $TNFR1^{-/-}$ mice, which have no mature FDCs with RML scrapie and observed no difference in disease pathogenesis between the TNF deficient mice and their wild type counterparts, suggesting that FDCs are not involved in scrapie pathogenesis (Klein, Frigg et al. 1997). This was further confirmed by Prinz *et al* who also found that $TNF^{-/-}$ mice developed scrapie at the same time as their WT counterparts. Furthermore, although PrP^{Sc} was absent in the spleen, they found high accumulation in the lymph nodes of these animals (Prinz, Montrasio et al. 2002). However, they also discovered PrP^{Sc} accumulation in macrophages in the lymph nodes of these mice, suggesting that unactivated macrophages may act as a replication site in the absence of FDCs (Prinz, Montrasio et al. 2002). Studies by Oldstone *et al* using the RML scrapie strain in $LT\alpha^{-/-}$ and $LT\beta^{-/-}$ mice analysed the role of FDCs after oral and i.p. scrapie inoculation. In

these studies, all of the LT$\alpha^{-/-}$ mice developed disease, whereas the lack of FDCs led to impaired scrapie pathogenesis in the LT$\beta^{-/-}$ mice. It was suggested that scrapie replication may be dependent on some other unknown cell type dependent on LTβ (Oldstone, Race et al. 2002). However, it should be mentioned that high doses of RML Scrapie were used for inoculation in these studies, so it may be that the need for an amplification stage in the spleen in unnecessary for neuroinvasion to proceed after high dose of infection. The discrepancies between various experiments using same strains have been put down to various factors such as TSE agent load and route of inoculation, however the reasons behind these differences will not be fully understood until the true nature of the TSE agent is discovered.

1.6 Thesis aims

From studies published so far, it has been determined that PrP^{C} expressing FDCs are required for the successful pathogenesis of many TSE agent strains within the lymphoid tissue (McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, McGovern et al. 2002; Prinz, Montrasio et al. 2002; Mabbott, Young et al. 2003). However in the models used so far, it is possible that FDCs acquire accumulation of PrP^{Sc} on their surface after scrapie agent replication on other neuronal, stromal or endothelial cells within the lymphoid tissue. To definitively determine the role of FDCs in scrapie pathogenesis, a model is required where PrP^{C} expression can be manipulated exclusively on the FDC network.

The aims of this thesis were to create and characterise transgenic mouse models that allow the manipulation of PrP^{C} expression specifically on FDCs and subsequently to

infect these animals with scrapie to determine the specific role of the FDCs in TSE pathogenesis within the lymphoid tissue. The hypothesis was explored that if FDCs are simply accumulating the TSE agent on their surface after replication on another cell type, the accumulation of the TSE agent will occur on PrP^C deficient FDCs when all other cell types within the lymphoid tissue retain PrP^C expression. If however, the FDCs actively replicate the TSE agent in the lymphoid tissue, accumulation of PrP^d will not occur on the PrP^C deficient FDCs. Additionally, if FDCs are responsible for replicating the TSE agent within the lymphoid tissue, then PrP^C expression exclusively on FDCs should be sufficient to allow replication of the TSE agent within the lymphoid tissue.

1.7 Transgenic mouse lines used in this thesis

1.7.1 Cre-LoxP Model

To create a model which allows manipulation of PrP^C specifically on FDCs, the Cre-LoxP sytem of targeted gene deletion was used. The Cre-LoxP system is a model that can be used to generate transgenic animals in which gene expression is tissue- or cell-type specific. This occurs through the use of the Cre-recombinase enzyme that cuts out or inverts any DNA that is flanked by LoxP sites i.e. "floxed" DNA. In non-Cre expressing cells the target DNA remains unchanged. This system can be made specific by inserting the Cre-recombinase after a tissue- or cell type- specific promoter (Kos 2004). LoxP is short for *Locus of crossover P* and these sites are 34bp DNA sequences that are used to flank the target DNA. They contain an 8bp core that determines the "directionality" of the target DNA and this is flanked by 13bp pallindromic sequences. Cre-recombinase is a 38 KDa cyclisation recombination

recombinase enzyme that creates the recombination of the DNA between LoxP sites. It is a member of the integrase family of recombinases and is coded for by bacteriophage P1 (Kos 2004). When Cre is activated, it creates a transient DNA-protein covalent linkage to bring the two LoxP sites together and catalyses a site-specific recombination event. Depending on the orientation of the two LoxP sites, the DNA will either be inverted or excised. If the LoxP sites are inverted, i.e. are in opposite directions, the DNA segment between undergoes inversion and the two LoxP sites remain in their position in the DNA. However if the two LoxP sites are in the same direction, Cre excises the target DNA segment and a single LoxP site remains (Kos 2004).

1.7.2 The *CD21-Cre* mouse line

The *CD21-cre* mouse line was chosen to create a model which allows manipulation of PrP^{C} expression exclusively on FDCs. CD21 is also known as complement receptor 2 (CR2) and was thought to be expressed solely on FDCs and mature B lymphocytes (Reynes, Aubert et al. 1985; Liu, Xu et al. 1997; Takahashi, Kozono et al. 1997). There are two types of CD21- a long and a short isoform- which are expressed on FDCs and B cells respectively. CD21 has a high affinity for the complement fragments C3dg and C3d and on FDCs, will bind antigens opsonised by these (Liu, Xu et al. 1997). On B lymphocytes, CD21 acts as a co-receptor for the B cell receptor (BCR), where the binding of specific antigen opsonised by complement components decreases the threshold level for signalling through the BCR. However, in humans, expression of CD21 has also been reported on a subpopulation of immature thymocytes (Tsoukas and Lambris 1988; Wagner and Hansch 2006), peripheral T

lymphocytes (Morgan, Marchbank et al. 2005) and on cervical epithelium (Sixbey, Lemon et al. 1986). Within the mouse, expression has also been reported on a small population of $CD4^+$ T lymphocytes within the MLN, activated granulocytes and mast cells (Gray and Skarvall 1988; Gray, Kosco et al. 1991; Andrasfalvy, Prechl et al. 2002; Heggebo, Gonzalez et al. 2003; Llewelyn, Hewitt et al. 2004). The expression of CD21 appears to more widespread than initially reported, however all non-FDC expression of CD21 is found on bone marrow derived cells. This is confirmed using data from the BioGPS database which stores microarray data of gene expression profiles from various mouse and human tissues. Analysis of CD21 (*Cr2*) expression using the BioGPS database confirms there is no non-lymphoid expression of CD21 reported (Fig 1.3). To restrict Cre expression in the *CD21-cre* mouse exclusively to FDCs, animals are lethally γ-irradiated and reconstituted with non-Cre expressing bone marrow.

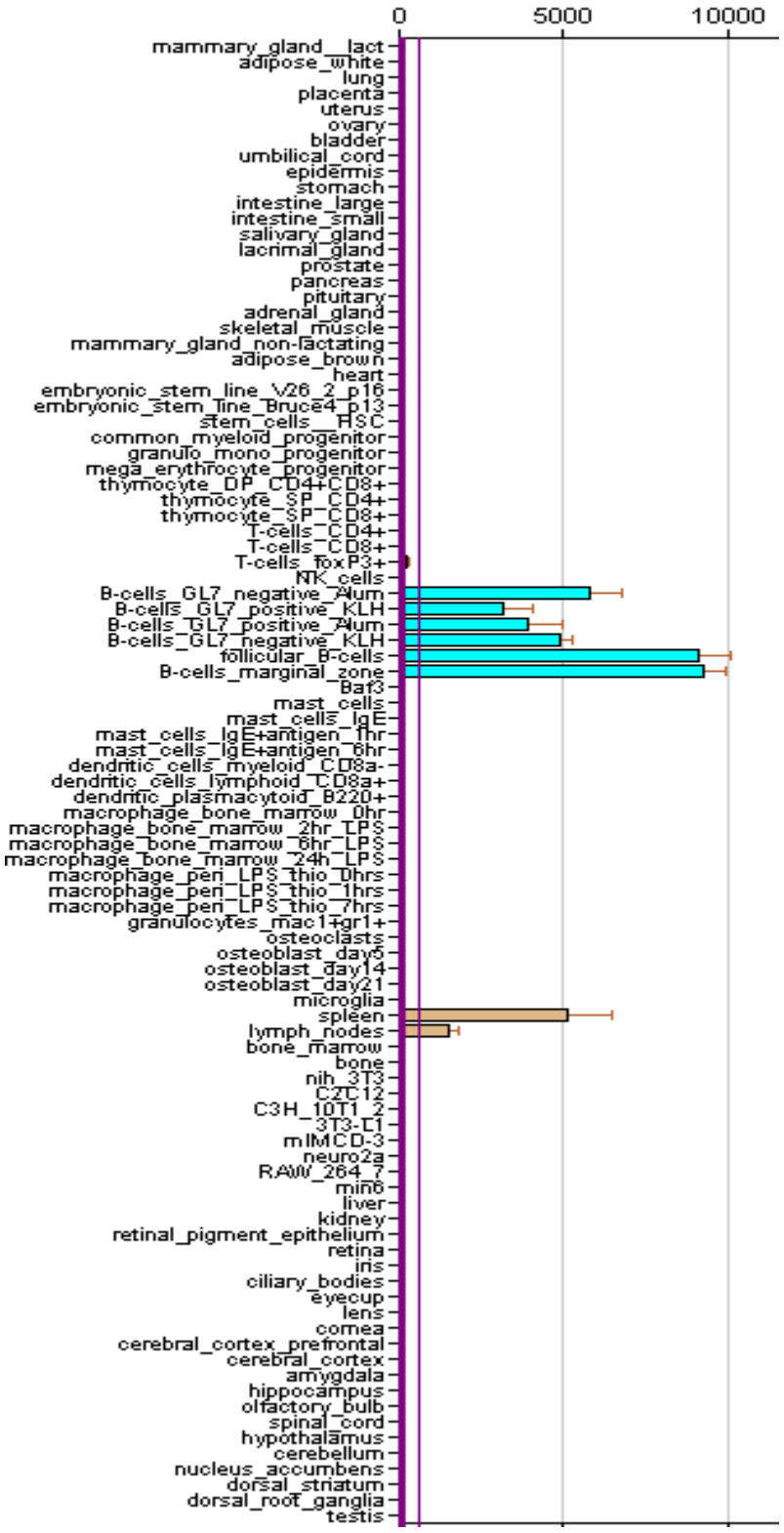


Figure 1.3 BioGPS expression profiles of *Cr2* in the mouse
Expression profiles of *Cr2* from BioGPS database (http://biogps.gnf.org) which stores microarray data from various murine tissues and cell lines. In agreement with publication data, *Cr2* expression is restricted to the lymphoid tissues on FDCs and bone marrow-derived cells.

CHAPTER 2

Materials and methods

2. Materials and methods

2.1 Production of mouse lines

2.1.1 *CD21-cre* mice

CD21-cre mice were kindly provided by Klaus Rajewski (CBR Institute for Biomedical Research) and were created by targeted insertion of CD21/35 bacterial artificial chromosome (BAC) clone containing a Cre expression cassette. Homologous recombination of *Cre* into the BAC was confirmed by Southern Blot and used for microinjection into fertilized oocytes (Kraus, Alimzhanov et al. 2004). *CD21-cre* mice are on a C57BL/6 background.

2.1.2. *CD21-creROSA26* mice

The *CD21-creROSA26* reporter strain was created at the Roslin Institute by crossing the *CD21-cre* mice with a *ROSA26* reporter strain (a kind gift from Yuko Fujiwara and Stuart H. Orkin, Howard Hughes Medical Institute, Harvard Med School, Massachusetts, USA). The *ROSA26* mice have a floxed 1.3-kb BglII–EcoRI ‘‘stopper’’ fragment from plasmid cAct-XstopXnZ (Fig 2.1), followed by the *LacZ* gene, a β-galactosidase coding sequence, inserted into the *ROSA26* locus. The *ROSA26* locus is ubiquitously expressed during embryonic development and in adult cells. Cell-specific expression of Cre causes removal of the floxed stop cassette and β-galactosidase expression is switched on in that cell type. (Mao, Fujiwara et al. 1999). The progeny of this cross were genotyped and selected for *Cre* and *LacZ* (ROSA26)

expression by PCR analysis of genomic DNA from tail snips. Age matched, Cre-negative *ROSA26* litter mates were used to control for background β-galactosidase staining.

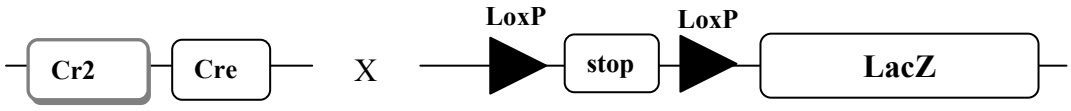


Fig 2.1 *CD21-creROSA26* mice
Cre is expressed under the *Cr2* promoter region. Expression of Cre causes excision of a floxed stop cassette, switching on expression of the LacZ gene which is a β-galactosidase coding region.

2.1.3 *CD21-cre*PrP$^{fl/fl}$ mice

The *CD21-cre*PrP$^{fl/fl}$ mouse strain was created by crossing the *CD21-cre* mice with mice which has the *Prnp* ORF flanked by *loxP* sites i.e. "floxed" (termed PrP $^{fl/fl}$) (Tuzi, Clarke et al. 2004). The ORF of the murine *Prnp* gene is contained within exon 3. The PrP$^{fl/fl}$ mice are on a 129/Ola background and have *loxP* sites flanking *Prnp* exon 3 created by homologous recombination with a PrP$^{fl/fl}$ targeting vector. Cell-specific Cre activation removes exon 3 of *Prnp* thus switching off PrP^{C} expression. The progeny mice were genotyped and selected for *Cre* and *Prnp^{fl}* expression.

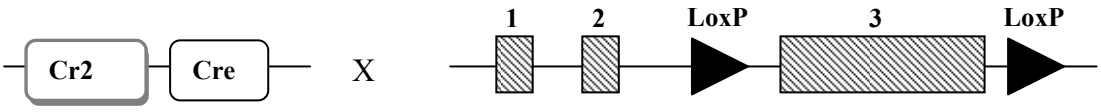


Figure 2.2 *CD21-cre*PrP$^{fl/fl}$ mice
Cre is expressed under the *Cr2* promoter region. Expression of Cre causes excision of the floxed exon 3 of the *Prnp* gene, which contains the protein coding region. Consequently, PrP^{C} expression is switched off in Cre-expressing cells.

2.1.4 *CD21-cre*PrP$^{fl/-}$ mice

Non promoter-specific recombination of the floxed *Prnp* ORF was detected in the *CD21-cre*PrP$^{fl/fl}$. Multiple generations of the *CD21-cre* line crossed with the floxed line on a mixed genetic background may have led to the *Cr2* promoter losing control of Cre expression. As a consequence, these mice were not used for subsequent scrapie experiments. To compensate for this, the *CD21-cre* mice were bred onto a *Prnp* null background by crossing with a PrP$^{-/-}$ mouse (Manson, Clarke, et al 1994). This PrP$^{-/-}$ mouse is on a 129/Ola background and was created by gene targeting a PrP DNA fragment construct containing a neomycin resistance gene into exon 3 of the *Prnp* gene, disrupting the protein coding region for PrP^{C}. These *CD21-cre*PrP$^{-/-}$ mice were then crossed with the PrP$^{fl/fl}$ line. Animals were selected by genotyping for *Cre*, *Prnp^{null}* and *Prnp^{fl}* and the resulting *CD21-cre*PrP$^{fl/-}$ mice produced were used in subsequent scrapie experiments. Age matched Cre-negative littermates were used as controls and bone marrow donors in experiments, where indicated.

2.1.5 *CD21-cre*PrP$^{stop/-}$ mice

The *CD21-cre*PrP$^{stop/-}$ strain was created by crossing *CD21-cre*PrP$^{-/-}$ mice with PrP$^{stop/-}$ mice (Tuzi, Clarke et al. 2004). The PrP^{stop} mice contain a floxed *β-geo* cassette inserted into intron 2 of the *Prnp* gene, upstream of the coding region. Insertion of this *β-geo* cassette prevents the expression of PrP^{C}, however site specific expression of Cre removes the cassette, switching on PrP^{C} expression. The PrP $^{stop/-}$ mice are also on a 129/Ola background. Animals were selected by genotyping for Cre, *Prnp^{null} and Prnp^{stop}* and the resulting *CD21-cre*PrP$^{stop/-}$ mice were used in subsequent

scrapie experiments. Age-matched Cre-negative littermates were used as controls and bone marrow donors in experiments, where indicated.

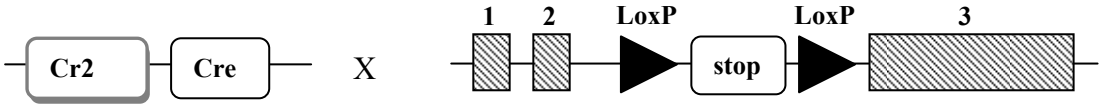


Figure 2.3 *CD21-cre*PrP$^{stop/-}$ mice
Cre is expressed under the *Cr2* promoter region. Expression of Cre causes excision of a floxed stop cassette inserted before exon 3 of the *Prnp* gene, which contains the protein coding region. Consequently, PrP^{C} expression is switched on in Cre-expressing cells.

2.1.6 Genotyping animals

Tail snips of *CD21-creROSA26* mice were lysed in 800µl of proteinase K (PK)-containing buffer (0.3 M sodium acetate, 0.1% SDS, 0.1M Tris pH8, 1 mM EDTA, 7mg/ml PK) and cleaned by addition of 600µl phenol chloroform followed by centrifugation. The supernatants were removed and added to 600µl of isopropanol and 20ul of 3M sodium acetate to pellet DNA. DNA pellets were then cleaned by centrifugation with 70% ethanol and resuspended in 100µl distilled water. Resulting DNA samples were analysed for *Cre* and *LacZ* expression using the primers in the table below (Table 2.1). PCR products were viewed by running on a 1% agarose gel, containing 0.001% ethidium bromide (Sigma).

For *CD21-cre*PrP$^{fl/fl}$, *CD21-cre*PrP$^{-/-}$, *CD21-cre* PrP$^{fl/-}$, *CD21-cre*PrP$^{stop/-}$ strains, DNA was prepared from tailsnips, earpunches, blood, tail or spleen using a DNeasy

blood and tissue kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

CD21-cre $PrP^{fl/fl}$ DNA samples were genotyped for *Cre* and $Prnp^{fl}$ using primers listed in Table 2.1. PCR product for Cre samples were viewed by running on a 1% agarose gel containing 0.001% ethidium bromide producing a band of 786 base pairs (bp).

$Prnp^{fl}$ product was run on a 2% agarose gel containing 0.001% ethidium bromide resulting in a band of 210 bp. $Prnp^{WT}$ could also be detected as a band of 167 bp which demonstrated whether the sample was homozygous or heterozygous for $Prnp^{fl}$. Animals were subsequently genotyped for $Prnp^{fl}$ with or without recombination of the floxed exon 3 ($Prnp^{fl(R)}$). To do this, the $Prnp^{fl}$ primers were used with the addition of an extra primer, *vitro Cre B*, to detect any recombination of the floxed exon. Full recombination resulted in one band of 344 bp whereas partial recombination resulted in both the recombined band of 344 bp and the $Prnp^{fl}$ band at 210 bp. $Prnp^{WT}$ was also detected in as a band of 167 bp as in the $Prnp^{fl}$ PCR.

CD21-cre $PrP^{-/-}$ DNA samples were genotyped for *Cre* as previously described. Samples were also genotyped for $Prnp^{null}$ and $Prnp^{WT}$ using primers listed in Table 2.1. PCR product was run on a 1% agarose gel containing 0.002% gelred (Biotium, Cambridge Biosciences Ltd, Cambridge, UK) and produced bands of 1.2 Kbp and 600 bp respectively.

CD21-cre PrP$^{fl/-}$ DNA samples were genotyped for *Cre*, *Prnp*^{WT} and *Prnp*^{null} as above and also *Prnp*$^{fl(R)}$ as in the genotyping of *CD21-cre*PrP$^{fl/fl}$ DNA samples with primers listed in Table 2.1.

CD21-cre PrP$^{stop/-}$ DNA samples were genotyped for *Cre*, *Prnp*^{WT} and *Prnp*^{null} as previously described. In addition, these samples were genotyped for *Prnp*^{stop} to detect the floxed STOP cassette and any recombination of the floxed STOP DNA using the primers listed in Table 2.1. This produced a band of 1 Kbp for the floxed stop cassette (*Prnp*^{stop}) and a band of 840bp for recombined *Prnp*^{stop} (*Prnp*$^{stop(R)}$).

2.1.7 Production of bone marrow chimeras

Where indicated, mice were lethally γ-irradiated with 950 rads from a Caesium^{137} source (Gravatom Engineering Systems Ltd, UK). Mice were reconstituted with bone marrow from age and sex matched animals 24 h post-irradiation. Bone marrow preparations were made as single cell suspensions in Hank's balanced salt solution (HBSS; GIBCO Life Technologies, Paisley, UK) from the femurs and tibias of selected donor mice. Bone marrow suspensions contained between 3x 10^7- 4x 10^7 viable cells per 1ml. Mice were reconstituted by injection of 0.1ml of bone marrow suspension into the tail vein. Animals were left for 100 days before experimental analysis to allow removal of long-lived B lymphocyte populations and replacement with the donor bone marrow.

2.1.8 Animal Housing

All protocols using experimental mice were approved by the Neuropathogenesis Unit's Protocols and Ethics Committee and conducted according to the regulations of the Home Office. Experimental work was carried out under project license number 60/3983 held by Dr. Karen Brown to investigate immune system influences on TSE pathogenesis. All mouse strains used were kept in a conventional animal housing unit under specific pathogen free conditions. Bone marrow chimeric mice were housed in individually ventilated cages.

2.2 Characterisation of *CD21-creROSA26* animals

2.2.1 Detection of β-galactosidase

Tissues from *CD21-creROSA26* animals were dissected into ice cold phosphate buffered saline pH 7.4 (PBS) and fixed for one hour in *LacZ* fixative [paraformaldehyde-gluteraldehyde (2% paraformaldehyde, 0.2% gluteraldehyde, PBS), 0.02% Nonidet P40, 0.01% sodium deoxycholate, 5mM EGTA, 2mM $MgCl_2$]. Tissues were then given three 20 minute washes in *LacZ* wash buffer (0.02% Nonidet P40, 0.01% Sodium deoxycholate, 2mM $MgCl_2$, 1X PBS pH 7.4). After washing, tissues were incubated in 15% sucrose in PBS overnight followed by a further overnight incubation in 30% sucrose in PBS. Tissues were subsequently embedded in Tissue-Tek® O.C.T. Compound™ (Bayer PLC, Newbury, UK) and frozen using isopentane at the temperature of liquid nitrogen. Serial sections of 8μm were cut on a cryostat and processed further for X-gal staining and immunohistochemistry.

To detect β-galactosidase, sections were incubated in *LacZ* staining solution [1X PBS pH 7.4 1mg/ml X-gal (Glycosynth, Warrington, UK), 0.02% Nonidet P40, 0.01% sodium deoxycholate, 2mM $MgCl_2$, 5mM potassium ferricyanide, 5mM potassium ferrocyanide] at 37°C overnight and protected from light. Staining reaction was stopped by washing for 5 min in *LacZ* wash buffer followed by distilled water for 1 min. Sections were counterstained with nuclear fast red (Vector Laboratories, Peterborough, UK), dehydrated and mounted in Pertex (CellPath, Powys, UK).

2.2.2 Immunohistochemistry (IHC) of frozen tissues

Sections (thickness 8μm) were cut from tissues fixed for β-galactosidase detection (Section 2.2.1) using a cryostat. Sections were fixed in acetone for 10 minutes before addition of a species specific normal serum block followed by primary antibodies as listed in Table 2.2. For light microscopy, a species specific biotin-conjugated secondary antibody was added. This was detected by alkaline phosphatase (AP) coupled to the avidin–biotin complex (Vector Laboratories) using Vector Red as a substrate (Vector Laboratories) and counterstained with haematoxylin. Light microscopy was carried out on a Nikon Eclipse E800 microscope (Nikon U.K. Ltd, Surrey, U.K.) For fluorescent microscopy, species specific secondary antibodies conjugated to alexa-fluor 488, 594 or 647 were added and detected by confocal microscopy. All fluorescent microscopy was carried out on a Zeiss LSM5 confocal microscope (Zeiss, Welwyn Garden City, UK)

PCR	Forward Primer(s)	Reverse Primer(s)	Product Size
Cre	**CreScreen 1** CGAGTGATGAGGTTC GCAAGAACC	**CreScreen 3** GCTAAGTGCCTTCT CTACACCTGC	786 bp
LacZ	**LACZ1ROSA26** TACCACAGCGGATGG TTCGG	**LACZ2ROSA26** GTGGTGGTTATGCC GATCGC	300 bp
Prnp^{fl}	**3'orfR1** GCCGACATCAGTCC ACATAG	**5'orfR1** GGTTGACGCCATG ACTTTC	*Prnp*^{fl} 210 bp *Prnp*^{WT}167 bp
Prnp$^{fl(R)}$	**Vitro CreB** AATGGTTAAACTTTC GTTAAGGAT	**3'orfR1 and 5'orfR1** As previous	*Prnp*$^{fl(R)}$ 344 bp *Prnp*^{fl} 210 bp *Prnp*^{WT}167 bp
Prnp^{null}	**Null A1** GCCATCACGAGATTT CGATT	**Null A2** ATCCCACGATCAGG AAGATG	1.2 Kbp
Prnp^{WT}	**PrP 44** TCATCCCACGATCAG GAAGATGAG	**PrP45** ATGGCGAACCTTGG CTACTGGCTG	600 bp
Prnp^{stop}	**STP1** ACAAATGTGGTATGG CTGATTATG **WTS1** TACCACGAAGTCCGG GATAG	**STP2** ATGATGATTGAACA AGATGGATTG **WTS2** GGCAGAGGCTAAGG ACAACA	*Prnp*^{stop} 1Kbp *Prnp*$^{stop(R)}$ 840 bp *Prnp*^{WT} 750Kbp

Table 2.1 Primers used in PCR genotyping of animals
Details of oligonucleotide primers used for PCRs to genotype transgenic animals during breeding to produce mouse lines detailed in section 2.1.

2.2.3 Characterisation of spleen and blood cells from *CD21-creROSA26* animals by flow cytometry

Single cell suspensions of spleens were prepared by passing cells through a cell strainer in 1ml of fluorescence- activated cell sorting (FACS) buffer [GIBCO 1xPBS pH 7.4 (Invitrogen, Paisley, UK) 2% Foetal calf serum (Sigma, Dorset, UK)]. Blood was collected into heparinised tubes to prevent clotting and added to FACS buffer. Cell suspension were spun at 2000 rpm for 10 min at 4°C and resulting cell pellets were re-suspended in red blood cell lysis buffer (Sigma) for 5 minutes. Viable cells were counted in a 1:10 dilution in trypan blue (GIBCO) and adjusted to 1 X 10^6 viable cells per 50 µl. Samples of $1x10^6$ cells were blocked using 1µl Seroblock FcR rat anti-mouse CD16/32 (AbD Serotech, Oxford, UK) and incubated for 30 minutes with an appropriate dilution of primary antibody (Table 2.3) at 4˚C.

Samples labelled with directly conjugated primary antibodies were transferred to FACS tubes for analysis. Samples labelled with biotin-conjugated primary antibodies were incubated for 30 min in a 1:200 dilution of streptavidin-conjugated alexa 594 or 488. Samples labelled with unconjugated primary antibodies were incubated with a 1:200 dilution of the species specific secondary antibody conjugated to a fluorochrome. To detect intracellular antigens, such as β-galactosidase, samples were permeabilised using a BD fixation/ Permeabilisation kit (BD bioscience, Oxford, UK). After labelling cell surface antigens, cells were added to BD Fixation/ Permeabilisation solution. Cells were incubated in the primary antibody for 30 minutes according to the manufacturer's instructions. Species specific, isotype controls were used in all experiments to detect any background fluorescence. A

FACS Scan flow cytometer (Becton Dickinson, Oxford, UK) was used to analyse cells with the lymphoid cells gated for by forward and side scatter.

2.3 Characterisation of *CD21-cre*PrP$^{fl/-}$ and *CD21-cre*PrP$^{stop/-}$ mouse lines

2.3.1 PCR analysis of bone marrow chimeric mice

Blood, tail and spleen samples were taken from transgenic mouse lines and snap frozen until DNA extraction was performed. DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Samples were genotyped for *Cre*, *Prnp*^{null}, *Prnp*$^{fl(R)}$ and *Prnp*^{stop} as described previously (2.1.5). Differences in gene expression between blood and spleen/ tail were used to demonstrate the chimera status of animals and confirm whether specific Cre-mediated DNA recombination was occurring in stromal, haematopoietic or both compartments.

2.3.2 Immunohistochemistry of frozen tissues

Tissues were snap frozen in liquid nitrogen, embedded in Tissue-Tek® O.C.T. Compound™ (Bayer PLC, Newbury, UK) and 8µm sections were cut using a cryostat. IHC analysis was carried out for light and fluorescent microscopy as previously described (2.2.2) using the antibodies listed in Table 2.2. Light and fluorescent microscopy was carried out as detailed previously (2.2.2)

2.3.3 ImageJ analysis of confocal images

Confocal images were analysed and converted to data using the program ImageJ (http://rsbweb.nih.gov/ij/index.html). This program processes images in Java to create data that were used to analyse the total area of specific immunostaining and the co-localisation of proteins. The accuracy of analysing images in this way was previously validated by comparing manual counts to automated computer generated data (Inman, Rees et al. 2005). This method of analysis has considerable advantages because it ensures consistency over analysis of images and is faster and more accurate than observational analysis. Furthermore, it allows statistical analysis and data to be extracted from images to prove true co-localisation of proteins as opposed to co-localisation due to the random association of fluorochromes on an image.

ImageJ was used to determine and compare the area occupied by FDC networks in spleen sections from each of the transgenic mouse lines when compared to wild type mice. To do this spleen sections were stained using the rat-anti-mouse CD35 antibody clone 8C12 as described previously (2.3.2). Five areas of each spleen were imaged using the Zeiss confocal microscope at x200 magnification. ImageJ was used to convert images to grayscale and measure the number of pixels positively labelled with the CD35 antibody. This enabled calculation of the average area of FDC per image.

Co-localisation of proteins was also assessed using ImageJ. Firstly, the multiple colour backgrounds macro (Appendix I) was used over 2 images from each of the animals. A line is drawn through the image and the macro measures the intensity of the colour over that line. This enables a threshold for background staining to be determined for each colour in the image. The multiple colour analysis macro

(Appendix II) is then used to measure the number of black and coloured pixels in the image, using the previously estimated threshold to eliminate the count of any background. These values were then used to work out the total area of staining for each antibody used and determine the amount of co-localisation

2.3.4 Fluorescence-activated cell sorting (FACS) analysis

Flow cytometry was used to determine PrP^C expression status of $CD21^+$ lymphocytes. Cells were extracted from both spleen and blood to demonstrate the chimera status of animals. Single cell suspensions from spleen and blood were prepared as described previously (2.2.3) and labelled with 1B3 and CD21/35 (Table 2.3). $CD21^+$ B lymphocytes were gated and assessed for PrP^C expression status using a FACS Scan flow cytometer (Becton and Dickinson).

2.3.5 *In-vivo* analysis of immune complex trapping by FDCs

Animals were given intra-peritoneal injections of 100 µl of preformed rabbit peroxidise-anti-peroxidase (PAP) immune complex (Sigma) and culled 48 h later. Spleens were snap frozen and 8µm sections were cut on a cryostat. Sections were blocked with normal goat serum and incubated with a goat-anti-rabbit antibody conjugated to alexa-fluor 555. After washing, sections were blocked with normal mouse serum and then incubated with biotin-conjugated rat-anti-CD35. Immunolabelling was detected using a streptavidin-alexa 488 conjugate. Images were taken on the Zeiss confocal microscope and were analysed for co-localisation using the ImageJ macro described previously (2.3.3)

Antigen	Primary Antibody	Source	Secondary Antibody	Source
CD21/CD35 (CR2/CR1)	Rat anti-CD21/35 Clone 7G6	BD Biosciences PharMingen	Biotinylated mouse-anti-rat IgG	Jackson Laboratories
PrP^c	Rabbit anti- PrP Polyclonal 1B3	Roslin Institute (Farquhar, Somerville et al. 1989)	Biotinylated goat anti-rabbit IgG	Jackson Laboratories
B220	Rat anti-CD45R Biotin conjugated CloneRA36A2	Caltag Laboratories	Streptavidin-alexa fluor conjugate	Invitrogen Life Technologies
Protein gene product 9.5 (Pan-neuronal marker)	Rabbit anti- PGP 9.5 Polyclonal	DakoCytomation	Biotinylated goat anti-rabbit IgG	Jackson Laboratories
Ubiquitin hydrolase (HuC, HuD and Hel-N1	Mouse anti-human HuC/D Clone 16A11 Neuronal protein	Invitrogen Life Technologies	Goat anti-mouse IgG_{2B} alexa 488	Invitrogen Life Technologies
bβ - galactosidase	Rabbit anti-β-galactosidase fusion protein Polyclonal	eBioscience Hatfield, U.K.	Goat anti-rabbit IgG-alexa 594	Invitrogen Life Technologies
CD35	Rat anti-CD35 biotin conjugated Clone 8C12	BD Biosciences PharMingen	Streptavidin-alexa fluor conjugate	Invitrogen Life Technologies
Milk fat globule EGF Factor 8 (MFGE-8)	Hamster anti-MFGE-8 Clone 18A2-G10	MBL International Woburn, MA, U.S.A.	Goat anti-hamster IgG-alexa 488	Invitrogen Life Technologies
Complement component C4 bound to FDC surface	Rat anti- FDC Clone FDC-M2	ImmunoKontact AMS Biotechnology, Abington, UK	Biotinylated mouse anti- rat IgG	Jackson Laboratories
Tyrosine Hydroxylase (TH)	Sheep anti-mouse TH Polyclonal	Millipore (Chemicon) Durham, U.K.	Donkey-anti-sheep Ig-alexa 488	Invitrogen Life Technologies
CD3- alexa 488	1/100 Clone 17A2	Biolegend U.K (Cambridge Bioscience)		
CD1d-biotin	1/100 Clone 1B1	Insight Biotechnology Middlesex, U.K.	Streptavidin-alexa fluor conjugate	Invitrogen Life Technologies
CD11c-biotin	1/100 Clone HL3	BD Biosciences Pharmingen	Streptavidin-alexa fluor conjugate	Invitrogen Life Technologies

Table 2.2 Detection antibodies used for immunohistochemical analysis of frozen sections

Antigen	Primary Antibody	Source	Control Antibody	Source
CD21/35	Rat anti-CD21/35-PE Clone 7G6	E Bioscience	Rat IgG_{2a}- PE	E Bioscience
CD19	Rat anti-CD19- FITC Clone 6D5	Invitrogen Life Technologies	Rat IgG_{2a}-FITC	Invitrogen Life Technologies
CD3	Rat anti -CD3-biotin Clone KT3	AbD Serotech	Rat IgG_{2a}-biotin	AbD Serotech
β-galactosidase	Rabbit anti-β-galactosidase-FITC Polyclonal	AbD Serotech	Rabbit IgG_{2a}-FITC	AbD Serotech
PrP	1B3 Polyclonal	Roslin Institute (Farquhar, Somerville et al. 1989)	Goat anti Rabbit IgG-FITC	Sigma-Aldrich

Table 2.3 Detection antibodies used for flow cytometric analysis
Details of antibodies used for immunolabelling of lymphocytes isolated from spleen and blood for detection by flow cytometry

2.4 Experimental analysis of scrapie infected animals

2.4.1 Inoculation with the scrapie agent

All animals were injected with the ME7 strain of the scrapie agent either intra-peritoneally (ip) or intra-cranially (ic). This strain was originally isolated from a spleen of a Suffolk sheep with natural scrapie and was passaged into Moredun random-bred mice via intra-gastric inoculation followed by passage via ic inoculation in Moredun random bred mice (Zlotnik and Rennie 1963). This was subsequently passaged nine times via ic inoculation in C57BL/Dk mice at the Neuropathogenesis Unit in Edinburgh. This strain has been extensively used as a mouse model of TSE disease and pathogenesis has been thoroughly characterised in inbred mouse lines (Bruce, Boyle et al. 2002). For ic inoculation, mice were anaesthetised with 3% fluorine gas in oxygen. Mice were injected in the right mid temporal cortex with 20 μl of 1% (wt/vol) scrapie-infected brain homogenate in physiological saline. For i.p. inoculation, 20μl of 1% (wt/vol) scrapie-infected brain homogenate was injected into the peritoneal cavity.

2.4.2 Immunohistochemical analysis of paraffin-embedded tissues

Spleens and brains from scrapie-infected mice were fixed in 2% periodate-lysine-paraformaldehyde (PLP) (0.1M sodium periodate, 0.075M D-L lysine, 2% paraformaldehyde in 0.05M PBS) and embedded in paraffin wax. Sections of 6μm in thickness were cut on a microtome. For detection of PrP in brains and spleens, sections were deparaffinised and autoclaved at 121˚C for 15 minutes. Sections were

immersed in 98% formic acid for 10 minutes followed by incubation with primary antibodies 1B3or 6H4 (Table 2.4) overnight at room temperature. Biotin-conjugated, species specific, secondary antibodies were incubated for one hour. PrP in the spleen was detected using avidin-biotin complex conjugated to AP (Vector Labs) and visualised using Vector Red. PrP in the brain was detected using avidin-biotin complex conjugated to horseradish peroxidise (HRP, Vector Labs) which was visualised using 3,3'-diaminobenzadine (DAB, Sigma). Spleens were also labelled for FDCs and B lymphocytes using antibodies listed in Table 2.4 and visualised using AP-Vector red. Brains were also labelled for activated astrocytes and microglia and visualised using AP-Vector red (Table 2.4).

2.4.3 Paraffin-embedded tissue (PET) blots

Antibodies used to detect PrP^{Sc} are polyclonal and can detect both PrP^{C} and PrP^{Sc}. To ensure labelling in infected spleens was the disease-associated PrP^{Sc}, paraffin-embedded tissue blots were used. During this procedure, treatment with PK destroys any cellular PrP^{C}, leaving only the pK-resistant PrP^{Sc} if present. Section of 6μm thickness were cut onto nitrocellulose membrane Trans-blot transfer medium (Bio-Rad Laboratories, Hertfordshire, U.K.) and dried overnight at 55˚C. Sections were deparaffinised and incubated overnight in 20 μg/ml pK (Sigma) in PK digest buffer (10 mM/0.01 M Tris pH 7.8, 100 mM/0.01 M Sodium Chloride, 0.1% Brij). Protein was denatured in 3mol/L guanidine isothiocyanate (GndSCN) for 10 minutes at room temperature followed by a block with 2% Casein western blotting reagent (Roch, Welwyn Garden City, U.K.) in Tris-buffered saline-Tween (TBS-tween; 10 mM/0.01 M Tris pH 7.8, 100 mM/0.01 M $NaCl_2$, 0.5% Tween 20). Primary polyclonal rabbit-

anti PrP, antiserum 1B3 (Neuropathogenesis Unit, Edinburgh) was added in a 1/4000 dilution in blocking buffer and incubated for 2 hours at room temperature. Bound 1B3 was detected using goat anti-rabbit conjugated to AP (Jacksons Laboratories) and visualised using Nitro-blue tetrazolium chloride- 5-bromo, 4-chloro, 3'-indolyphosphate P-toluidine salt (NBT/BCIP) tablets (Sigma) dissolved in distilled water. Sections were imaged using a Lecia WLD MZ8 stereo light microscope (Lecia, Milton Keynes, UK)

Antigen	Primary antibody	Pre-treatment	Source
PrP^{Sc}	Rabbit anti-PrP Clone 1B3	Autoclave 121˚C and Formic acid	Roslin Institute (Farquhar, Somerville et al. 1989)
PrP^{Sc}	Mouse anti-PrP Clone 6H4	Autoclave 121˚C and Formic acid	Prionics, Zurich, Switzerland
FDCs (CD35)	Rat anti-CD35 Clone 8C12	Citrate buffer	BD Biosciences PharMingen
B lymphocytes (B220)	Rat anti-CD45R Biotin conjugated Clone RA36A2	None	Caltag Laboratories
Microglia	Iba1	Citrate buffer	Abcam, Cambridge, U.K.
Activated astrocytes	Glial fibrillary acidic protein (GFAP) Polyclonal	None	Dako, Camebridgeshire, UK

Table 2.4 Detection antibodies used for immunohistochemical analysis of paraffin-embedded sections
Details of antibodies used for immunolabelling of tissue sections from paraffin embedded tissues.

CHAPTER 3

Characterisation of the *CD21-cre* mouse using a *ROSA26* reporter strain

3.1 Abstract

Follicular dendritic cells (FDCs) are considered to be important sites of scrapie agent accumulation in lymphoid tissues after peripheral exposure. However, models used so far have not been able to distinguish the role of the FDCs from that of all other stromal and lymphoid cells found within the lymphoid tissue. The *Cr2* gene encodes complement receptor 2 (CR2) also known as CD21, is reported to be expressed only on FDCs and mature B lymphocytes. In this chapter, the *CD21-cre* mouse was investigated as a possible tool for creating an FDC-specific transgenic mouse model. This was achieved by crossing the *CD21-cre* mouse line with the *ROSA26* reporter line, resulting in β-galactosidase expression in cells where Cre is activated. To restrict Cre expression exclusively to FDCs, animals were lethally γ-irradiated and reconstituted with non-transgenic WT bone marrow. Cellular localisation of Cre expression in tissues was determined by the histological detection of β-galactosidase expression. Cre was successfully activated in FDCs and B lymphocytes in the *CD21-creROSA26* mouse line. Irradiation and reconstitution of *CD21-creROSA26* mice with WT bone marrow successfully restricted Cre expression to the FDCs in lymphoid tissues. Data from the *CD21-creROSA26* model suggests that the *CD21-cre* mice reconstituted with WT bone marrow are a useful tool to manipulate gene expression exclusively on FDCs.

3.2 Introduction

After peripheral infection, for example oral or ip exposure, many TSE agents undergo a stage of intense replication in the spleen and lymph nodes accompanied by high deposition of PrP^{Sc} (Fraser and Dickinson 1970; Clarke and Haig 1971; van Keulen, Schreuder et al. 1996). FDCs reside in the B cell follicles of peripheral lymphoid tissue and express relatively high amounts of PrP^{C} on their surface, expression of which is essential for scrapie pathogenesis (Klein, Frigg et al. 1998; Brown, Stewart et al. 1999). In the lymphoid tissue of scrapie affected mice strong immunolabelling for PrP^{Sc} is associated with FDCs (McBride, Eikelenboom et al. 1992; Bruce, Brown et al. 2000). To confirm this finding, analysis of infected lymphoid tissue at the ultra-structural level showed high accumulation of PrP^{Sc} on the FDC surface and around the dendrites (Jeffrey, McGovern et al. 2000). Studies using mice depleted of FDCs have shown significant increases in incubation time, delayed pathogenesis and reduced susceptibility (Mabbot 2000; Montrasio 2000; Mabbot 2002; Oldstone 2002; Mabbott, Young et al. 2003). However, in the models used so far, there has been no way to dissociate PrP^{C} expression on FDCs from stromal, neural and lymphoid cells within the lymphoid tissue. Therefore it is possible that FDCs themselves do not actively replicate the TSE agent, but instead accumulate it on their surface after replication on another cell type. Thus, to definitively determine the role of the FDCs in peripheral TSE pathogenesis, a model is required where manipulation of PrP^{C} expression is exclusively restricted to the FDC network.

The *Cre-loxP* system can be used to generate transgenic animals in which gene expression is tissue- or cell type-specific. This occurs through the use of a Cre-

recombinase (Cre) that cuts out or inverts any DNA that is flanked by *loxP* sites i.e. "floxed" DNA. Whereas, in non-Cre expressing cells the target DNA remains unchanged. This system can be made specific by inserting Cre after a tissue- or cell type- specific promoter (Kos 2004). In this chapter, the *CD21-cre* mouse (Kraus, Alimzhanov et al. 2004) is tested as a possible method of manipulating gene expression exclusively on FDCs. In this strain, Cre is inserted after the *Cr2* promoter, which encodes CD21. CD21 is considered to be restricted to FDCs and mature B lymphocytes. (Reynes 1985; Liu 1997; Takahashi, Kozono et al. 1997; Heggebo, Press et al. 2002). However, in humans, expression of CD21 has also been reported on a subpopulation of immature thymocytes (Tsoukas and Lambris 1988; Wagner and Hansch 2006), peripheral T lymphocytes (Fox, Jewell et al. 2006) and on human cervical epithelium (Sixbey, Lemon et al. 1986) . Expression has also been reported within the mouse on a population of $CD4^+$ T lymphocytes found within the mesenteric lymph node, activated granulocytes and mucosal mast cells (Gray and Skarvall 1988; Gray and Matzinger 1991; Andrasfalvy, Prechl et al. 2002; Heggebo, Gonzalez et al. 2003). In this study, to overcome expression of Cre in lymphocytes, the mice will be exposed to lethal γ-irradiation, which will deplete host bone marrow-derived cells, and subsequently reconstituted with donor, non-Cre-expressing, bone marrow. As a consequence, all Cre-expressing host-derived lymphocytes are depleted and replaced with the non-transgenic WT donor lymphocytes restricting Cre-expression to the FDCs.

Next, to determine the cellular localisation of Cre activation in *CD21-cre* mice, this line was crossed with a Rosa reporter strain. The *ROSA26* line contains a floxed STOP cassette in front of the *LacZ* gene, which contains a β-galactosidase coding

region (Mao, Fujiwara et al. 1999). In cells which activate Cre, the STOP cassette is removed by the activity of Cre and β-galactosidase expression is switched on. Expression of β-galactosidase can be detected using X-gal, a substrate of β-galactosidae. Digestion of X-gal by β-galactosidase produces a blue product which can be used to histologically locate where β-galactosidase is expressed. Lymphoid and a large variety of non-lymphoid tissues from both the *CD21-creROSA26* line and *CD21-creROSA26* animals that were lethally γ-irradiated and reconstituted with non transgenic bone marrow from WT mice were then assessed to determine the cellular sites of Cre expression. This analysis confirmed that Cre is activated in the FDCs and mature B lymphocytes in *CD21-cre* mice and this expression is restricted to the FDC network after irradiation and reconstitution with WT bone marrow. Therefore it was concluded that *CD21-cre* mice will be a useful tool to manipulate gene expression exclusively in FDCs.

3.3 Results

3.3.1 Production of *CD21-creROSA26* mice

To determine the cellular localisation of Cre expression in the *CD21-cre* mouse line, *CD21-cre* mice were first crossed with the *ROSA26* reporter line which induces the expression of β-galactosidase in cells which activate Cre. Animals were genotyped by PCR and chosen based on expression of the Cre and Rosa (*LacZ*) transgenes. To restrict Cre expression to FDCs, animals were given whole body, lethal, ionising radiation to remove all host lymphocytes and were subsequently reconstituted with non-transgenic, WT bone marrow (termed WT →*CD21-creROSA26* mice). Cellular expression of Cre driven by the *Cr2* promoter was assessed in animals with and without bone marrow reconstitution (Fig. 3.1). In the *CD21-creROSA26* animals, expression of Cre induces production of the bacterial enzyme β-galactosidase in Cre expressing cells. An X-gal stain for β-galactosidase expression is used to locate β-galactosidase and assess Cre activity. Animals expressing only the *ROSA26* transgene were used to control for any background β-galactosidase staining that may be present.

3.3.2 Irradiation of *CD21-creROSA26* mice followed by reconstitution with WT bone marrow successfully removes Cre expression in $CD21^+$ B-lymphocytes

Cellular sites of Cre expression in tissues from *CD21-creROSA26* mice was assessed using an X-gal stain to detect β-galactosidase expression. X-gal (5-bromo, 4-chloro, 3-indolyl β-D-galactopyranoside) is a substrate of β-galactosidase. Tissue sections

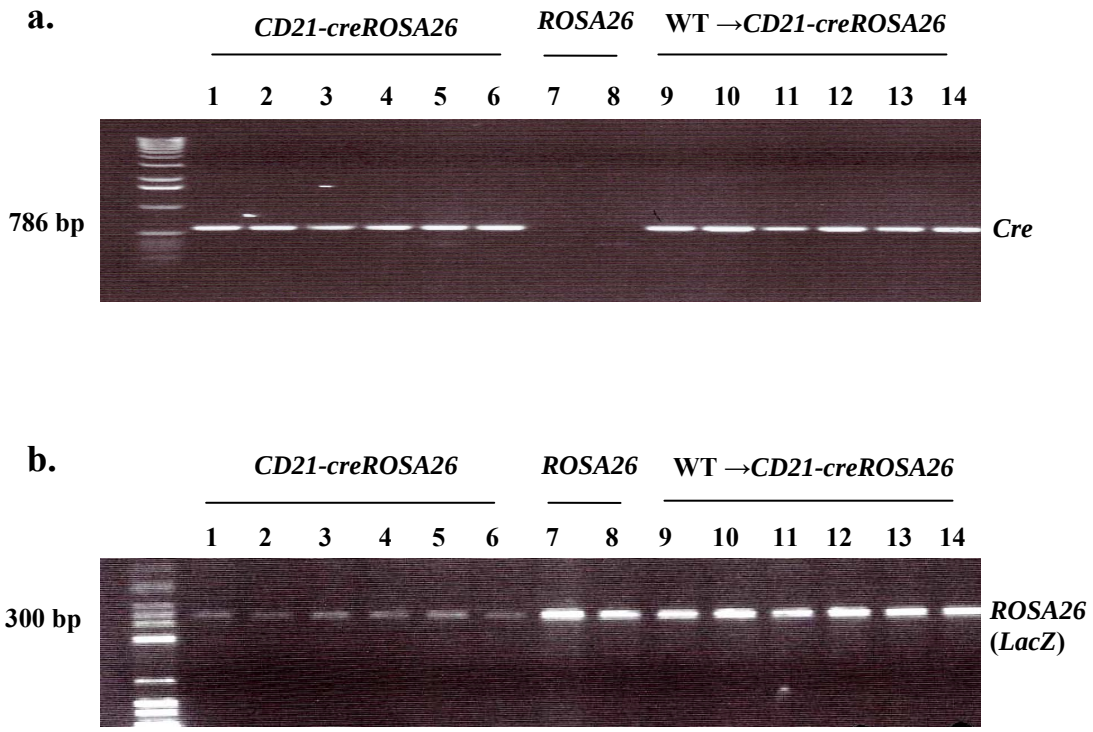


Figure 3.1 Confirmation of genotype of *CD21-creROSA26* mice
DNA was extracted from the tails of *CD21-creROSA26*, *ROSA26* and WT →*CD21-creROSA26* mice. A PCR analysis was used to confirm the presence of *Cre* and *LacZ* transgenes. This analysis confirmed that *CD21-creROSA26* (lanes 1-6) and WT →*CD21-creROSA26* (lanes 9-14) mice contained both the *Cre* and *LacZ* transgenes. As anticipated *ROSA26* mice (lanes 7-8) lacked the *Cre* transgene.

were immersed in X-gal solution which in Cre-expressing cells is catalysed by β-galactosidase to form a blue product that can be visualised using light microscopy.

B-lymphocytes and FDCs are closely compacted within the B lymphocyte follicle of lymphoid tissues. For this reason, it is difficult to distinguish β-galactosidase labelling of B lymphocytes in the follicle from that of FDC networks. However, labelling in the follicles of *CD21-creROSA26* mice which received irradiation and reconstitution with WT bone marrow was greatly reduced in comparison to labelling in follicles of untreated *CD21-creROSA26* animals (Fig 3.2). Therefore it can be concluded that irradiation and reconstitution with WT bone marrow successfully removes Cre-expressing $CD21^+$ B lymphocytes and restricts Cre expression to the FDC networks in the lymphoid tissues.

Single cell labelling of β-galactosidase was detected in the villi and isolated lymphoid follicles (ILFs) of the large and small intestine in tissues from *CD21-creROSA26* mice which did not receive any pre-treatment (Fig 3.5). This labelling was presumed to be intra-epithelial B lymphocytes and B lymphocytes in the capillaries, lymphatics and follicles of the intestine. However monoclonal antibodies against B lymphocyte markers were not sensitive enough to detect single cells after an X-gal stain. This type of single cell labelling was also demonstrated in the thymus and lung and was thought to be circulating lymphocytes in the capillaries of these organs. In *CD21-creROSA26* mice which received the irradiation and bone marrow reconstitution, labelling of single cells within these tissues was no longer detected. Therefore it can be concluded that irradiation and reconstitution with WT bone marrow successfully removes Cre-expressing $CD21^+$ circulating B lymphocytes.

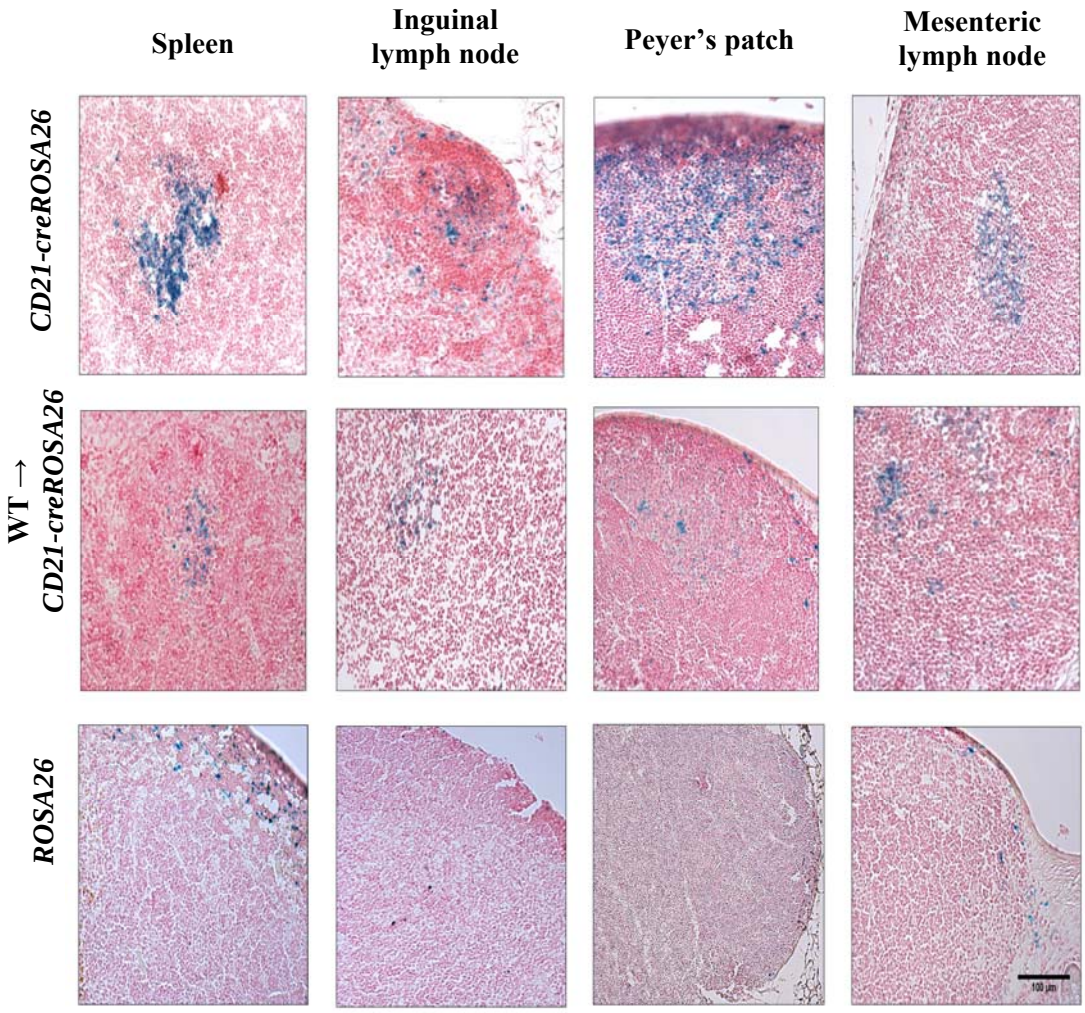


Figure 3.2 Irradiation of *CD21-creROSA26* mice and reconstitution with wild type bone marrow successfully removes Cre expressing B lymphocytes
Sections of lymphoid tissues from *CD21-creROSA26* and *CD21-creROSA26* given irradiation and WT bone marrow (WT → *CD21-creROSA26)* to remove all the transgenic $CD21^+$ B lymphocytes were stained for β-galactosidase (blue) expression using an X-gal staining solution and were counterstained with nuclear fast red (pink). Lymphoid tissues from Rosa mice containing the *LacZ* reporter gene insert but no *Cre* transgene were stained as negative controls. In all lymphoid tissues from Cre expressing animals, staining of β-galactosidase is found in the B-lymphocyte follicles which contain both FDCs and $CD21^+$ B lymphocytes. The levels of staining in lymphoid tissues from animals which received the irradiation and bone marrow graft is greatly reduced in comparison to those that did not receive this pre-treatment. This suggests that β-galactosidase, and therefore Cre expression, has been removed from $CD21^+$ B lymphocytes in the lymphoid tissue. This implies that WT →*CD21-creROSA26* mice are a useful model to study FDC-restricted gene expression.

3.3.3 Cre is activated by the *Cr2* promoter in FDCs and CD21$^+$ B lymphocytes

The cellular localisation of Cre expression was assessed in the lymphoid tissues, including the spleen, mesenteric, axillary and inguinal lymph nodes, thymus, Peyers patches and isolated ILFs of the gut. CD21 expression was initially reported to be restricted to CD21$^+$ mature B lymphocytes and FDCs however, expression has since been reported in other bone marrow-derived cells in mice, including T lymphocytes, activated granulocytes and mast cells, however this expression was postulated to be removed by the irradiation and bone marrow reconstitution (Gray and Skarvall 1988; Gray and Matzinger 1991; Andrasfalvy, Prechl et al. 2002; Llewelyn, Hewitt et al. 2004). To determine whether Cre-mediated DNA recombination occurred in other cell types, various non-lymphoid tissues were also analysed. These included the heart, lung, liver, kidney, large intestine, small intestine, pancreas, tongue, epithelium (ear), muscle, sciatic nerve, spinal cord and brain. Results describing the detection of β-galactosidase activity in all tissues is summarised in Table 3.1

β-galactosidase activity was detected on FDC networks within the B lymphocyte follicles of all lymphoid tissues in *CD21-creROSA26* mice, both with and without pre-treatment of lethal irradiation and reconstitution with WT bone marrow (Fig 3.2). Subsequently, an X-gal stain followed by immunolabelling of B lymphocytes and/or FDCs was used to determine cellular location of the β-galactosidase. The β-galactosidase labelling was found in areas of the follicle which also showed positive immunolabelling for FDC and FDC/B lymphocyte markers demonstrating that Cre recombinase is effectively activated under the *Cr2* promoter in FDCs and CD21$^+$ B lymphocytes (Fig. 3.3)

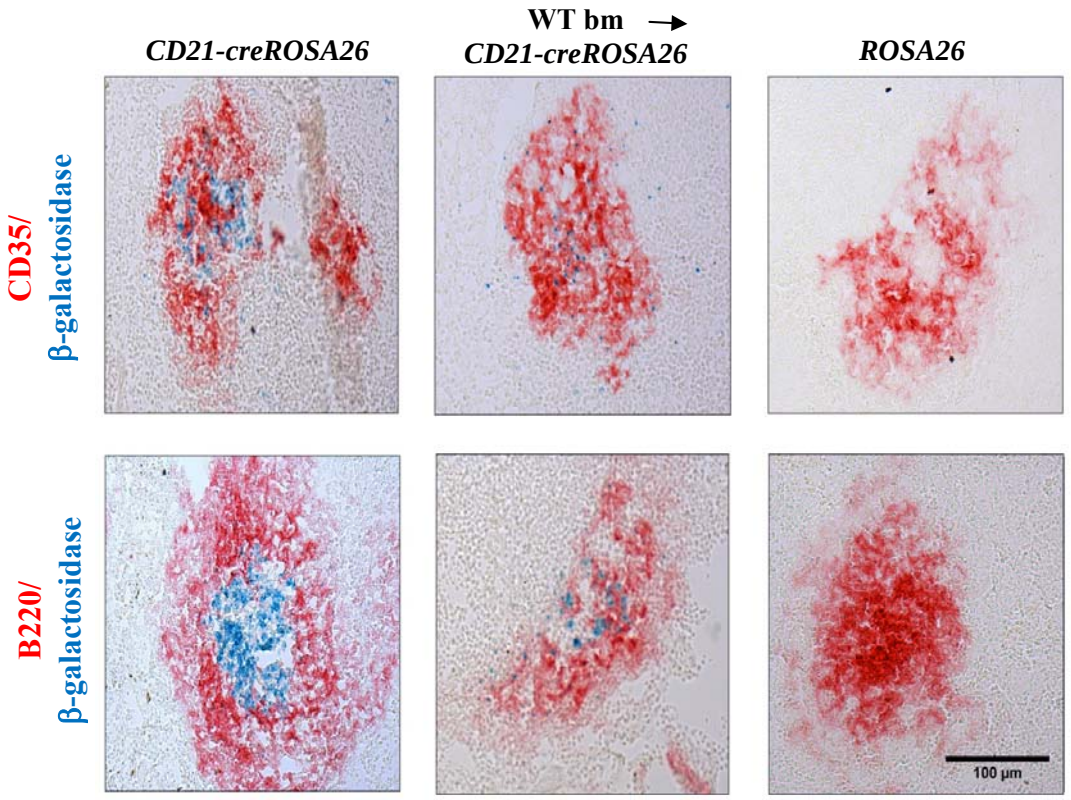


Figure 3.3 Cre is expressed within B lymphocyte follicles and FDC networks in *CD21-creROSA26* mice

Spleen sections from *CD21-creROSA26*, WT →*CD21-creROSA26* and *ROSA26* mice, were stained for β-galactosidase (blue) and B lymphocytes (red) or FDCs (red). Immunohistochemical detection of B lymphocytes using anti-B220 monoclonal antibodies and FDCs using anti CD35 monoclonal antibodies occurs in the same area of the follicle as β-galactosidase detected by an X-gal stain. This demonstrates that Cre is successfully expressed under the CD21 promoter in FDCs and CD21-expressing B lymphocytes. Scale bar 100µm.

Tissue	β-galactosidase staining	Notes
Spleen	present	On FDCs and B-lymphocytes. Background on capsule
Inguinal Lymph Node	present	On FDCs and B-lymphocytes. Background on capsule
Axillary Lymph Node	present	On FDCs and B-lymphocytes. Background on capsule
Mesenteric Lymph Node	present	On FDCs and B-lymphocytes. Background on capsule
Peyers Patches	present	On FDCs and B-lymphocytes. Background on capsule
Thymus	present	On B lymphocytes. Background at edge of tissue
Duodenum	present	On B lymphocytes and cells of plexi . Background in crypts, Brunners glands and contaminating intestinal bacteria
Jejunum	present	On B lymphocytes and cells of plexi . Background in crypts and contaminating intestinal bacteria
Ileum	present	On B lymphocytes and cells of plexi . Background in crypts and contaminating intestinal bacteria
Colon	present	On B lymphocytes and cells of plexi . Background in crypts and contaminating intestinal bacteria
Heart	absent	Background only
Lung	present	On B lymphocytes and background on mucosal surfaces
Liver	absent	No staining
Kidney	absent	Background only
Pancreas	absent	No staining
Ear	absent	No staining
Tongue	absent	No staining
Muscle	absent	No staining
Ovary, uterus,	absent	Background only
Testes,bladder	absent	Background only
Sciatic Nerve	absent	No staining
Brain	present	Staining of a population of cells in brain
Spinal Cord	absent	No staining

Table 3.1 Expression patterns of β-galactosidase in *CD21-creROSA26* mice
Summary of β-galactosidase staining found in tissues from 6 untreated *CD21-creROSA26* and 6 WT →*CD21-creROSA26* mice. Staining was concluded to be B-lymphocyte staining if it was present in the untreated group but absent in the group which received the irradiation and bone marrow. Staining was concluded to be background if it was also present in the same regions in tissues from *ROSA26* mice. Examples of this can be seen in Figure 3.5

3.3.4 Insertion and expression of the *Cre* transgene under the *Cr2* promoter has no toxic effect on CD21 expressing cells

Cre toxicity is a reported phenomenon that can occur in some *Cre* transgenic lines. In these cases, Cre recombinase targets sequences of DNA that are similar to its 24 bp target LoxP sites causing mis-recombination, DNA damage and death of the Cre-expressing cells (Schmidt-Supprian and Rajewsky 2007). Immunohistochemistry and flow cytometry was used to determine whether insertion of the *Cre* transgene has any adverse effects on CD21-expressing cells. Sections of spleen from *CD21-creROSA26* and WT →*CD21-creROSA26* mice were labelled with antibodies against FDCs and B lymphocytes and compared to spleen sections from WT and *ROSA26* controls. Immunohistochemical analysis showed no differences in FDC or B lymphocyte labelling between the transgenic animals and their WT counterparts (Fig. 3.4). In addition, $CD21^+$ B lymphocytes were isolated from spleen and counted by FACS analysis. FACS analysis of spleens from each mouse group showed no differences in either the number of $CD21^+$ or $CD19^+$ B lymphocytes (Fig. 3.5).

3.3.5 Detection of β-galactosidase expression in non-lymphoid tissues

Unexpectedly, β-galactosidase expression was detected in a population of cells in the ganglia of the myenteric and submucosal plexi of the large and small intestine. This staining only occurred in a small number of ganglia per section, however it was observed consistently in all of the animals (Fig 3.7). Practical difficulties were encountered in determining the precise nuclei with which the X-gal staining was associated with, possibly due to differential cellular packaging of β-galactosidase. Therefore, it could not be determined from the X-gal staining, whether this Cre-

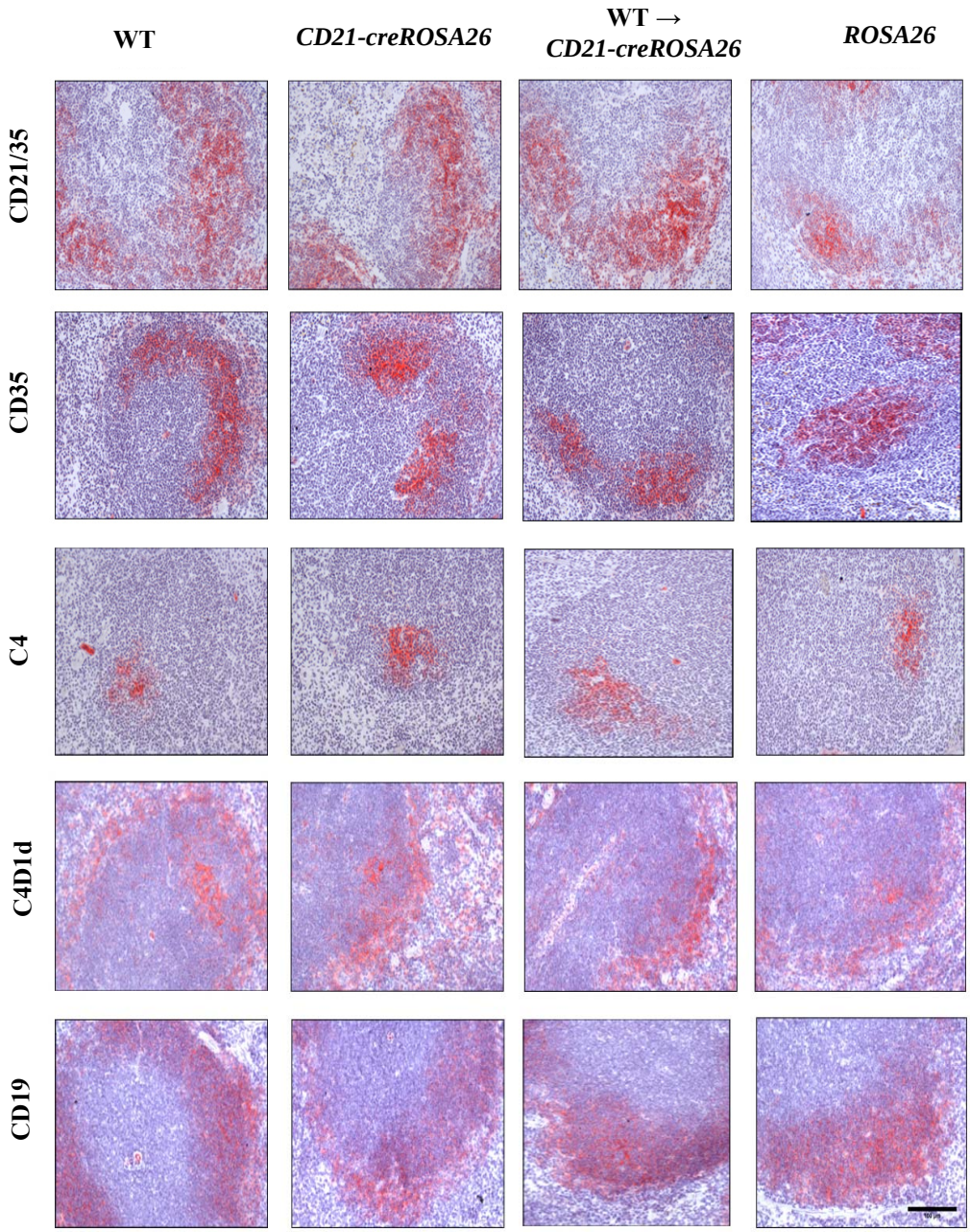


Figure 3.4 Expression of Cre under the *Cr2* promoter has no effect on FDCs and CD21$^+$ B lymphocyte numbers

Immunohistochemical detection of B lymphocytes (B220, CD19, CD1d, red) and FDCs (CD35, C4, red) in spleens from double transgenic mice, with and without irradiation and reconstitution with WT bone marrow, *ROSA26* and C57BL/6 lines. There is no observed difference in immunolabelling of B lymphocytes and FDCs of transgenic mice in comparison to C57BL/6 WT control line using a panel of anti-B lymphocyte and anti-FDC monoclonal antibodies. Scale bar 100 μm. Sections counterstained with haematoxylin, blue

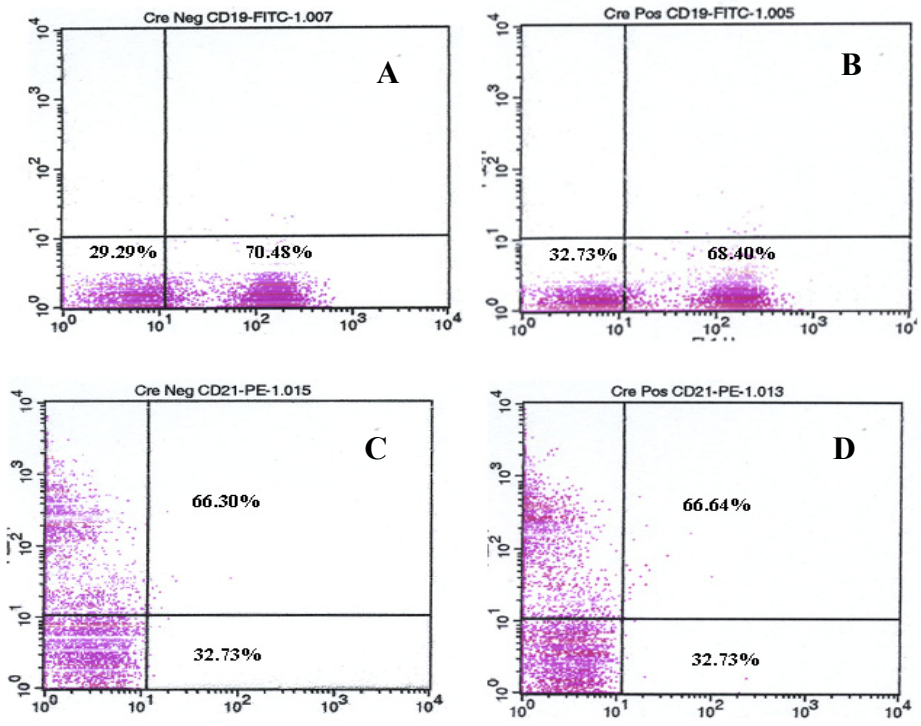
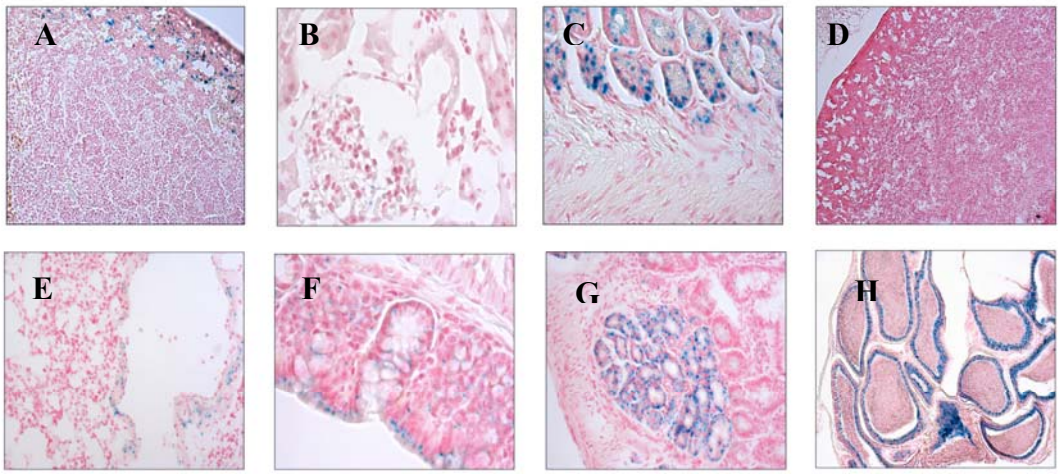


Figure 3.5 Expression of Cre under the *Cr2* promoter has no effect on CD21$^+$ B lymphocyte numbers

Detection of CD21 and CD19 on splenic lymphocytes from *CD21-creROSA26* and C57BL/6 mice. No difference was observed in the numbers of CD19$^+$ splenic B lymphocytes in *CD21-creROSA26* mice (B) in comparison to WT controls (A). There is also no difference in CD21 expression between the transgenic line (D) and WT control (C). These data suggest that insertion and expression of Cre under the *Cr2* promoter has no toxic effect on B lymphocytes numbers or the expression of CD21 or CD19.

a.



b.

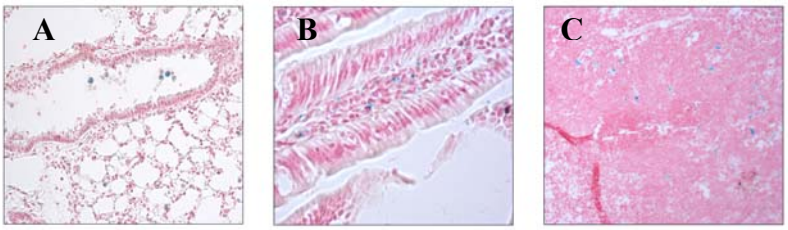


Fig 3.6 β-galactosidase detection in cells other than FDCs
Sections from various tissues of *CD21-creROSA26*, WT →*CD21-creROSA26* and ROSA26 transgenic animals were stained for β-galactosidase (blue) and counterstained with nuclear fast red (pink).

a. Background β-galactosidase staining was found in some tissues from Rosa animals containing no *Cre* transgene. These included the capsule of the spleen (A), the tubules and glomeruli of the kidney (B), paneth cells in the crypts of the intestines (C), the thymus (D), the brush borders of the bronchiole of the lung (E) and villi of large intestine (F), Brunner's glands in the duodenum and the uro-genital tract of both male and female animals (eppididymus pictured H) Original magnification D x100 A, E, F, G, H x200 B, C x400

b. Single cell β-galactosidase staining was detected in the lung (A), villi of intestine (B) and thymus (C) of *CD21-creROSA26* mice but not in WT →*CD21-creROSA26* mice. These were concluded to be circulating $CD21^+$ Cre-expressing B lymphocytes which were removed after irradiation and replaced with non-Cre-expressing WT bone marrow derived $CD21^+$ B lymphocytes. Original magnification C, x100 A, B x400

expression was in neuronal or glial cell populations within the ganglia. To clarify this, immunohistochemistry was attempted using an anti-β-galactosidase antibody along with an antibody against a pan-neuronal marker which recognises a neuronal protein named protein gene product 9.5 (PGP 9.5), to examine whether expression of these proteins were co-localised. Unfortunately, anti-β-galactosidase immunostaining using this antibody was not achieved even on positive control spleen and lymph node sections tested. An alternative method using an X-gal stain followed by immunohistochemistry for PGP 9.5 was tried instead (Fig 3.7). Although both staining of PGP 9.5 and X-gal occurred within the ganglia, it did not provide any further information on the cellular location of the X-gal staining.

The sciatic nerve and spinal cord did not show any activation of *Cre*. However β-galactosidase staining occurred in a population of large cells in the brain (Fig 3.7.). As with the co-localisation analysis on the plexi of the intestine, immunolabelling with anti-β-galactosidase antibodies was unsuccessful, therefore it is uncertain whether this staining is occurring within neuronal or glial cell populations. However, as irradiation and reconstitution with WT bone marrow did not remove this staining from both the intestine and the brain, it is likely that the *Cre* activation was within host-derived neurones rather than glial cells.

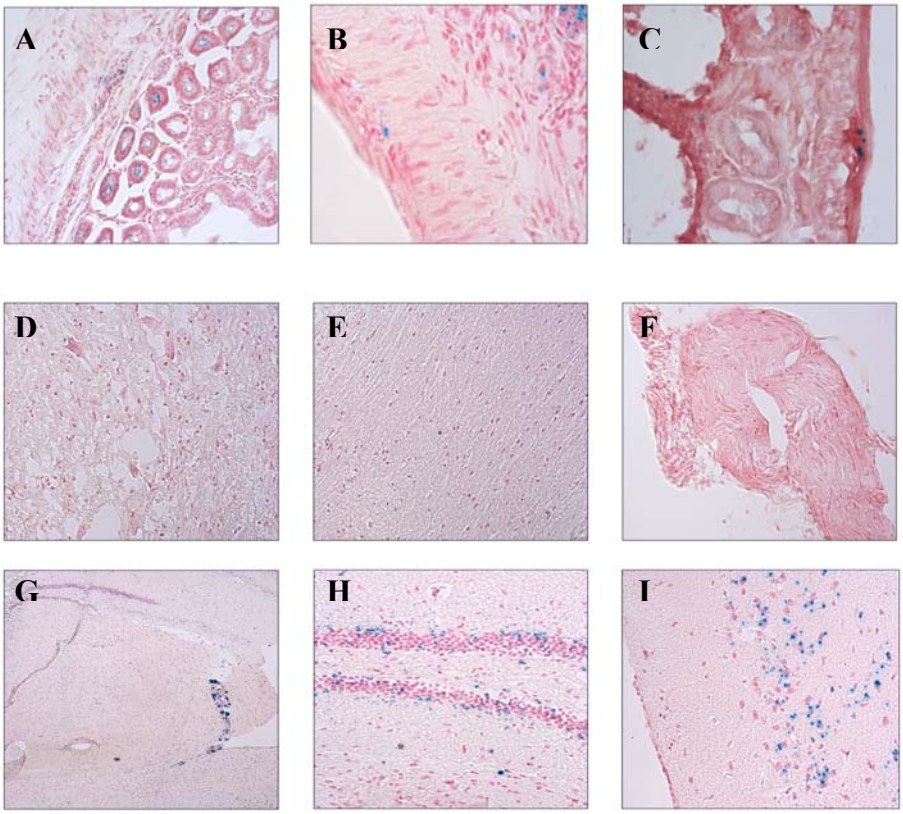


Fig 3.7 β-galactosidase labelling occurs in a population of cells in the plexi of intestine and the brain

A population of cells in the ganglia of both the submucosal (A) and myenteric (B) plexi of the intestine were found to express β-galactosidase (blue). This only occurred in a few ganglia per section of intestine, however it appeared consistently in each of the 12 animals analysed. Co-localisation analysis using anti-β-galactosidase antibodies and neuronal antibodies was unsuccessful (data not shown). Staining for β-galactosidase using an X-gal staining solution followed by immunolabelling of nerves using the monoclonal antibody PGP 9.5 (Red) (C) was not accurate enough to determine if this β-galactosidase was expressed in a neuronal or glial cell population within the ganglia.

Transverse (D) and longitudinal (E) sections of spinal cord were both clear of β-galactosidase labelling, as was the sciatic nerve (F).

However a population of cells in the brain which appeared in the optic tract (G), hippocampus, (H) and corpus collosum (I) also expressed β-galactosidase.

Sections are counterstained with nuclear fast red (pink). Original magnification A, C x100, B, D, E, F, G, H, I x200.

3.4 Discussion

In this chapter, the *CD21-cre* mouse was investigated as a possible model for restricting gene expression to FDCs. This was achieved by crossing the *CD21-cre* mouse with a *ROSA26* reporter strain which allows the detection of Cre activity via the production of β-galactosidase. The characterisation studies undertaken on this mouse line indicate that the *CD21-cre* model will be a useful tool to manipulate PrP^C expression specifically on FDCs and to study the role of the FDC network in TSE pathogenesis. Cre-expression under the *Cr2* promoter did not appear to affect lymphoid tissue structure as determined by immunolabelling of B lymphocytes and FDCs using a panel of monoclonal antibodies against various cell-specific markers. This is in accordance with Kraus et al (2004), who reported that insertion and expression of Cre under the *Cr2* promoter does not interfere with B lymphocyte development or numbers (Kraus, Alimzhanov et al. 2004).

The detection of β-galactosidase expression using the *ROSA26* reporter strain has shown that Cre-mediated DNA recombination is occurring efficiently on both FDCs and B lymphocytes. Although DNA recombination on B lymphocyte subsets was not examined, previous studies suggest that mature B lymphocytes express the highest levels of CD21, while immature and pro- and pre- B lymphocytes express low levels or none, respectively (Takahashi, Kozono et al. 1997). Studies on the *CD21-cre* line by Victoratos et al indicate that efficiency of this DNA recombination is 75% on B lymphocytes and 96% in FDCs (Victoratos, Lagnel et al. 2006). This was reflected in this characterisation study as all *CD21-creROSA26* animals showed efficient expression of β-galactosidase in the FDCs and B lymphocytes in a variety of lymphoid tissues tested.

Irradiation and reconstitution with WT bone marrow successfully restricted Cre expression to the FDC networks within the lymphoid tissue. The animals were lethally γ-irradiated (950 rads) and reconstituted with age and sex matched bone marrow 24 h later. Animals were maintained for 100 days post bone marrow transfer before analysis of tissues. Previous studies have shown that a minimum of 28 days is required for efficient re-differentiation of the FDC networks and repopulation of lymphocytes from donor bone marrow (Brown, Stewart et al. 1999). However, populations of long-lived B lymphocytes have also been reported, therefore animals were left for 100 days post irradiation and bone marrow transfer to ensure removal of the majority of host derived lymphocytes (Miller and Cole 1967).

Unfortunately FACS analysis of B lymphocytes using anti-β-galactosidase monoclonal antibodies was attempted for both cell surface and intracellular β-galactosidase but was unsuccessful. Fluorescent immunolabelling of tissue sections using the same antibodies also did not work. This is possibly because all tissues taken were fixed specifically for staining with X-gal in fixative which included detergents to permeabilise tissues. However when comparing levels of X-gal staining between *CD21-creROSA26* animals with *CD21-creROSA26* animals which were irradiated and given bone marrow, staining was greatly reduced in the irradiated animals suggesting that expression of β-galactosidase by B lymphocytes had been removed.

Staining of β-galactosidase in non-lymphoid tissues was also detected, for example in the kidney, lung, and intestine (Table 3.1). This is thought to be Cre-activation on circulating B lymphocytes as this staining was absent in *CD21-creROSA26* animals which were irradiated and re-grafted with WT bone marrow. In agreement with this,

Kraus et al performed analysis of Cre-mediated DNA recombination on B lymphocyte populations isolated from various tissues and found intermediate levels of recombination in the kidney and gut samples, confirming the presence of Cre-mediated DNA recombination in $CD21^+$ B lymphocytes found in non-lymphoid tissues (Kraus, Alimzhanov et al. 2004). As the irradiation and reconstitution with WT bone marrow removes all Cre expressing lymphocytes, CD21-expressing cells within these tissues should have no impact on the specificity of the model. Expression of CD21 has also been reported on activated granulocytes and mast cells in mice, however again, any Cre expression in these cell types will be eliminated by the irradiation and bone marrow reconstitution pre-treatment (Gray and Matzinger 1991; Ahmed and Gray 1996).

β-galactosidase expression on a small subset of cells in the thymus was also consistently detected. A population of immature human thymocytes has been reported to express CD21 and additionally in the mouse, CD21 expressing activated T lymphocytes have been reported (Gray and Skarvall 1988; Tsoukas and Lambris 1988; Llewelyn, Hewitt et al. 2004) . However, a study analysing CD21 expression in the mouse found that CD21 expressing cells in the thymus always co-localised with B lymphocyte markers, and suggested that these were the rare thymic B lymphocyte pool (Takahashi, Kozono et al. 1997). Again, this labelling was eliminated after irradiation and reconstitution with WT bone marrow.

Expression of β-galactosidase was detected in a population of neuronal or glial cells in the brain. β-galactosidase labelling was detected in large cells of the hippocampus, corpus collosum, dentate gyrus, coroid plexus and occurs diffusely throughout the

cortex. As monoclonal antibodies against β-galactosidase did not work on these tissues, it was not possible to do double immunolabelling and co-localisation analyses to determine which subset of cells were expressing Cre. This Cre-mediated DNA recombination was still present in animals which received irradiation and bone marrow reconstitution. The manipulation of gene expression in these cells in *CD21-cre* mice should have no impact in the early stages of TSE agent accumulation in the spleen as the agent does not reach the brain until the late stages of the incubation period. However, this may need to be taken into account for any animals that are left to progress to CNS disease as manipulation of PrP expression in these neurones may affect disease outcome.

Expression of β-galactosidase was also found in a population of cells within the ganglia of the myenteric and submucosal plexi of both the large and small intestine. For reasons stated previously, it was not possible to determine whether this staining occurred in a neuronal or glial cell population. These cells are reasonably close to the sites of initial prion accumulation in oral inoculation. Expression of β-galactosidase was only found in a few ganglia of these plexi per section of intestine but was expression was consistent in most of the animals. Sciatic nerve and spinal cord did not show any labelling for β-galactosidase.

A study by Gonzalez et al investigated the role of the enteric nervous system in oral inoculations with the murine BSE strain 301V. They observed strong labelling for PrP on both the myenteric and submucosal plexus of the inoculated mice, however, much of this was removed after PK treatment indicating that it was mainly the cellular PrP^{C} that was being detected (Gonzalez, Terry et al. 2005). Due to the detection of

Cre-mediated DNA recombination within the intestine, the ip route was chosen as the route of TSE agent exposure in future studies to avoid possible effects of Cre-mediated DNA recombination in enteric ganglia.

In conclusion, these data show the *CD21-cre* model will be a useful tool to manipulate *Prnp* expression exclusively on FDCs to determine the role of FDCs in scrapie pathogenesis after ip inoculation. Cre-mediated DNA recombination occurred efficiently on the FDC networks and mature B lymphocytes. Furthermore, irradiation and repopulation with non-Cre-expressing bone marrow successfully eliminated the expression of Cre on B-lymphocytes within the lymphoid tissues.

CHAPTER 4

Characterisation of the *CD21-cre*PrP$^{fl/-}$ mouse line

4.1 Abstract

Data from the *CD21-creRosa26* model (Chapter 3) suggests that *CD21-cre* mice reconstituted with WT bone marrow are a useful tool to manipulate gene expression exclusively on FDCs. PrP^C expressing FDCs are considered to be important sites of scrapie agent accumulation in lymphoid tissues after peripheral exposure. However, models used so far have not been able to definitively distinguish the role of the FDCs from that of all other stromal, lymphoid and neural cells found within the lymphoid tissue. For this reason, it is possible that rather than actively replicating the scrapie agent, FDCs may simply accumulate the agent after replication on another cell type. To generate a model which may be used to determine the specific role of FDCs in peripheral scrapie pathogenesis the *CD21-cre* mouse line was crossed with a line where the coding region of PrP^C is flanked by *loxP* sites ($PrP^{fl/fl}$) allowing PrP^C to be switched off under control of the *Cr2* promoter. The progeny *CD21-cre*PrP^{fl} mice were fully characterised to determine within which cells PrP^C was switched off and ensure that the transgenes had no additional effects on the lymphoid tissues that could have an impact on scrapie pathogenesis. Characterisation of the *CD21-cre*PrP^{fl} mouse demonstrated that PrP^C was efficiently and exclusively switched off on Cre-expressing FDCs. Furthermore, insertion and expression of the transgenes had no adverse effects on the microarchitecture of the lymphoid tissue or the number, area or functionality of FDCs. These data suggest that the *CD21-cre*PrP^{fl} mouse will be a useful tool to study the role of FDCs in scrapie pathogenesis.

4.2 Introduction

Expression of the cellular prion protein, PrP^{C}, is essential for efficient scrapie pathogenesis to occur (Bueler, Fischer et al. 1992; Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997; Klein, Frigg et al. 1998; Brown, Stewart et al. 1999; Wroe, Pal et al. 2006). In the lymphoid tissue of scrapie affected mice strong immunolabelling for PrP^{Sc} is associated with FDCs (McBride 1992; Bruce, Brown et al. 2000; Jeffrey, McGovern et al. 2000). Previous studies have shown that expression of PrP^{C} on FDCs is essential for scrapie pathogenesis to occur after peripheral exposure (Brown, Stewart et al. 1999). Previous data suggests that FDCs are responsible for replicating the scrapie agent within the lymphoid tissue prior to neuroinvasion and CNS disease. However in the models used so far, there has been no way to dissociate PrP^{C} expression on FDCs from all other stromal, neural and lymphoid cells within the lymphoid tissue. Therefore it is possible that FDCs themselves do not actively replicate the TSE agent, but instead accumulate it on their surface after replication on another cell type. Data in this thesis (Chapter 3) shows that the *CD21-creROSA26* mouse efficiently activated Cre-mediated DNA recombination in the FDCs and this could be made specific by lethal γ-irradiation and reconstitution with non-Cre expressing bone marrow. Therefore this model was crossed with a transgenic floxed PrP line to allow PrP^{C} to be switched off exclusively on FDCs.

The coding region for PrP^{C} is contained within exon 3 of the *Prnp* gene. In $PrP^{fl/fl}$ mice, exon 3 of *Prnp* is flanked by *loxP* sites i.e. "floxed" exon 3 (Tuzi, Clarke et al. 2004). This mouse strain was crossed with the *CD21-cre* mouse to allow PrP^{C} to be switched off under control of the *Cr2* promoter. Based on data from the *CD21-*

creROSA26 model, the *CD21-cre*PrP^{fl} mouse together with lethal γ-irradiation and reconstitution with non-Cre-expressing bone marrow, should allow PrP^{C} to be switched off exclusively on FDCs. In this chapter, the *CD21-cre*PrP^{fl} line was characterised to determine if PrP^{C} was successfully switched off on the FDC networks and that the transgenes had no additional affects on the lymphoid tissue. Analysis of the *CD21-cre*PrP^{fl} line showed that PrP^{C} was efficiently switched off on Cre-expressing FDCs however expression remained present on other non-CD21-expressing cells in the lymphoid tissues such as the nerves. Furthermore, there were no differences in the michroarchitecture and the number, area and function of FDCs in the spleen. From these data, it can be concluded that the *CD21-cre* PrP^{fl} line will be a useful tool to study scrapie pathogenesis when PrP^{C} has been switched off exclusively on FDCs.

4.3 Results

4.3.1 Production of *CD21-cre* $PrP^{fl/fl}$ mouse line

To switch of PrP^{C} in FDCs the *CD21-cre* line was crossed with the $PrP^{fl/fl}$ line, and progeny selected by screening for expression of *Cre* and $Prnp^{fl}$ by PCR. Progeny mice were then bred together, further selecting for *Cre* and $Prnp^{fl}$ expression until sufficient *CD21-cre*$PrP^{fl/fl}$ mice were obtained for use in characterisation studies. During this process, the results of the genotyping indicated there was a problem and DNA was extracted from a number of tissues to assess where $Prnp^{fl}$ DNA was being recombined in the animals. In *CD21-cre*$PrP^{fl/fl}$ animals, the PCR results should show partial recombination of $Prnp^{fl}$ DNA in the lymphoid tissues due to the presence of $CD21^{+}$ B lymphocytes and FDCs, and also a band for un-recombined $Prnp^{fl}$ due to the presence of non-CD21-expressing cells in the tissues. This is termed "partial recombination" of $Prnp^{fl}$ DNA. In the first mouse analysed, partial recombination of $Prnp^{fl}$ DNA was found in the spleen, mesenteric lymph node (MLN) and inguinal lymph node (ILN) as expected. However partial recombination was also detected in the liver, kidney, brain and ear but not in the heart or lung (Figure 4.1). This suggested that Cre-mediated DNA recombination was not occurring under control of the *Cr2* promoter. Tissues analysed from animals taken from subsequent rounds of breeding showed full recombination of the $Prnp^{fl}$ DNA with no un-recombined $Prnp^{fl}$ present (Figure 4.1). This spontaneous recombination of the $Prnp^{fl}$ DNA resulted in a *Prnp* deficient mouse as all $Prnp^{fl}$ DNA was recombined to remove exon 3 of the *Prnp* gene, switching off PrP^{C} expression in all cell types. As a consequence, this line could no longer be used in subsequent scrapie experiments.

4.3.2 Production of *CD21-cre*PrP $^{fl/null}$ mouse line

To compensate for the spontaneous recombination occurring in the $PrP^{fl/fl}$ homozygous mice, the *CD21-cre* line was subsequently bred as $Prnp^{floxed}$ heterozygotes on a $PrP^{-/-}$ background. To achieve this, the *CD21-cre* line was first crossed with a $PrP^{-/-}$ line. The resulting *CD21-cre*X$PrP^{-/-}$ mice were then crossed with the $PrP^{fl/fl}$ line and *CD21-cre*$PrP^{fl/-}$ progeny were used in subsequent experiments.

To switch off PrP^{C} exclusively in FDCs, *CD21-cre*$PrP^{fl/-}$ animals were lethally γ-irradiated and given bone marrow from age- and sex-matched Cre negative littermates (termed Cre^{-ve} →*CD21-cre*$PrP^{fl/-}$). Experimental control groups were also produced. These included, *CD21-cre*$PrP^{fl/-}$ mice given bone marrow from Cre^{+ve} littermates, (termed Cre^{+ve} →*CD21-cre*$PrP^{fl/-}$), in which PrP^{C} will be switched off in both FDCs and $CD21^{+}$ B lymphocytes. Also, $PrP^{fl/-}$ mice were given bone marrow from *CD21-cre* $PrP^{fl/-}$ mice, (termed Cre^{+ve} → $PrP^{fl/-}$), in which PrP^{C} is switched off on $CD21^{+}$ B lymphocytes only. The final control group was $PrP^{WT/-}$ mice given $PrP^{WT/-}$ bone marrow. The copy number of the *Prnp* gene has been shown to have a strong influence in scrapie incubation period, with $PrP^{WT/-}$ heterozygous mice having almost double the incubation time of $PrP^{WT/WT}$ homozygotes (Manson, Clarke et al. 1994b) Therefore, this control group was included due to the *CD21-cre*$PrP^{fl/-}$ experimental line having half levels of PrP^{C} expressed.

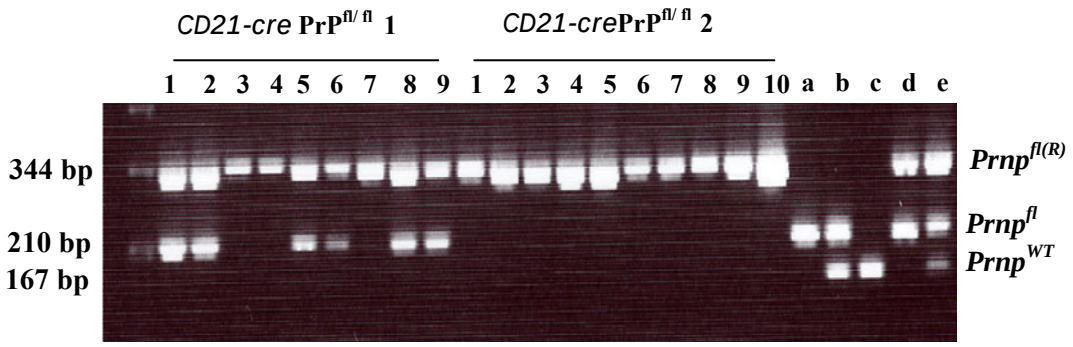


Figure 4.1 Spontaneous recombination of *Prnp*^{fl} DNA in *CD21-cre*PrP$^{fl/fl}$ mice
DNA was extracted from a variety of tissues from *CD21-cre*PrP$^{fl/\ fl}$ mice and genotyped by PCR to determine in which tissues *Prnp*^{fl} DNA was recombined. Tissues analysed included liver (1), kidney (2), heart (3), lung (4), spleen (5), ILN (6), MLN (7), brain (8), ear (9) and gut (10). PCR results show a band of 167 bp for *Prnp*^{WT} (c), a band of 210 bp for *Prnp*^{fl} (a), and a band of 344 bp for *Prnp*$^{fl(R)}$ recombined DNA. Heterozygous PrP$^{fl/WT}$ or PrP $^{fl/-}$ animals have bands at 167 bp and 210 bp (b). Homozygous PrP$^{fl/fl}$ animals with partial recombination of the *Prnp*^{fl} DNA show bands at 210bp and 344 bp (d) whereas heterozygous PrP$^{fl/WT}$ or PrP $^{fl/-}$ animals with partial recombination of the *Prnp*^{fl} DNA show all three bands (e).

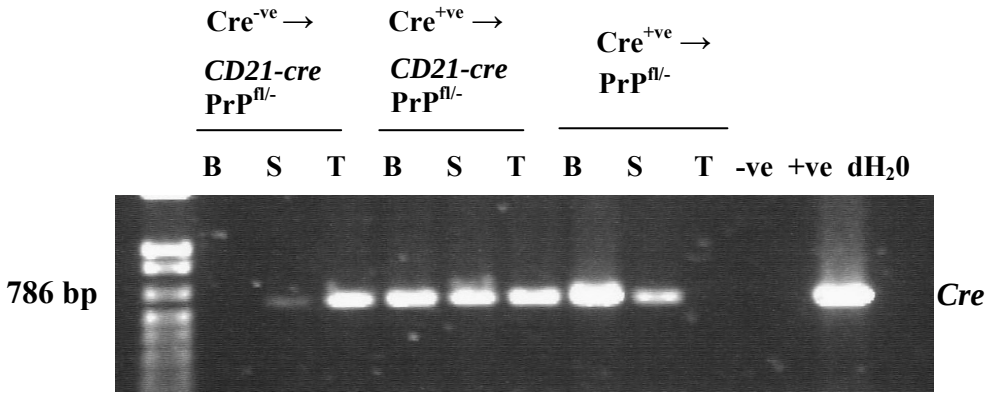
Tissues from the first animal assessed, *CD21-cre*PrP$^{fl/fl}$ 1, showed partial recombination of *Prnp*^{fl} DNA in the spleen and lymph nodes as expected, but also in the liver and kidney. Tissues from an animal taken after subsequent rounds of breeding, *CD21-cre*PrP$^{fl/fl}$ 2, showed full recombination of *Prnp*^{fl} DNA in all tissues. This demonstrates that the *Cr2* promoter has lost control of Cre-mediated recombination of *Prnp*^{fl} DNA. These animals are now PrP$^{-/-}$ as all *Prnp*^{fl} DNA has been recombined.

Bone marrow chimera status of experimental animal groups was confirmed by PCR analysis (Fig 4.2). DNA was extracted from blood, tail and spleen and was genotyped for both *Cre* and $Prnp^{fl}$ with or without Cre-mediated recombination of the $Prnp^{fl}$ DNA. This analysis confirmed that the host/ donor genotypes were as expected and also that $Prnp^{fl}$ DNA was only recombined in tissues where both the *Cre* transgene and CD21-expressing cells were present.

4.3.3 Insertion and expression of transgenes and bone marrow reconstitution have no effect on lymphoid tissue michroarcitecture

In addition to confirming that Cre-mediated recombination was restricted to CD21-expressing cells and was occurring efficiently, it was also essential to ensure that there were no other changes to the lymphoid tissue that could mediate an effect on scrapie pathogenesis. Spleens from all four groups of experimental mice were immunolabelled to detect B lymphocyte subsets, T lymphocytes, FDCs and dendritic cells and compared tissues from WT animals (Fig 4.3). No differences could be seen in intensity or location of immunolabelling for each cell type between WT and experimental spleens. It can be concluded that neither expression of the transgenes nor irradiation and bone marrow reconstitution has any significant effect on the michroarchitecture of the lymphoid tissues. Furthermore, spleen sections taken from *CD21-cre*$PrP^{fl/-}$, Cre^{-ve} $PrP^{fl/-}$ and WT 129/Ola mice were immunolabelled using an anti-CD35 MAb to detect the FDC networks. FDC networks in the spleen were manually counted and the area of FDC networks in the spleen was measured using ImageJ image analysis software (Inman, Rees et al. 2005). This quantification demonstrated that there were no significant differences in the number or area of FDC

a.



b.

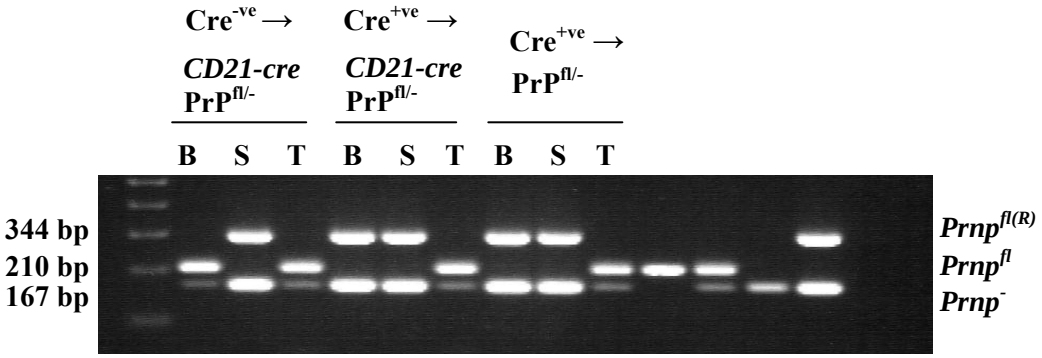


Figure 4.2 Genotypes of *CD21-cre*PrP$^{fl/-}$ animals used for characterisation and subsequent scrapie experiments

DNA was extracted from blood (B), spleen (S), and tail (T) from 6 animals from each of the transgenic lines to be used in subsequent scrapie experiments. One example of genotype results for Cre^{-ve} →*CD21-cre*PrP$^{fl/-}$, Cre^{+ve} →*CD21-cre*PrP$^{fl/-}$ and Cre^{+ve} →PrP$^{fl/-}$ lines is shown on the above gels.

a. The *Cre* transgene was present in the spleen and tail of *CD21-cre*PrP$^{fl/-}$ mice but was only present in the blood if Cre^{+ve} donor bone marrow had been given. PrP$^{fl/-}$ mice had no *Cre* transgene detected in tail DNA, however *Cre* was present in the spleen and blood due to Cre^{+ve} bone marrow given.

b. Tissues from all animals contained *Prnp^{fl}* and *Prnp^{-}* bands. However, recombined *Prnp$^{fl(R)}$* DNA was only detected in tissues where both the *Cre* transgene and CD21-expressing cells are present.

networks or differences in the number or area of FDC networks between transgenic mice and WT controls with P=0.221 and 0.091 respectively using a one-way ANOVA test (Fig 4.4). Taken together, these results show that insertion of the *Cre* transgene under the *Cr2* promoter has no adverse effects on the number and size of the FDC networks, or the general michroarchitecture of the surrounding lymphoid tissue.

4.3.4 PrP^C immunolabelling is removed on Cre-expressing FDCs

Spleens from all experimental and control lines were immunolabelled to detect PrP^C expression to ensure that PrP^C protein was specifically removed from the FDC network in animals that expressed the Cre transgene. Within the lymphoid tissues, PrP^C can be detected on FDCs and peripheral nerves using immunolabelling. Therefore, to determine if removal of PrP^C was specific to the FDC network, spleen sections were fluorescently immunolabelled for PrP^C, FDCs and peripheral nerves. Co-localisation of fluorescent labelling was then quantified using Image J software. Animals which expressed Cre showed removal of PrP^C specifically on the FDC networks but not on the peripheral nerves (Fig 4.5). Quantification of co-localised pixels showed that the co-localisation detected was significant co-localisation ($P=5.4 \times 10^{-30}$ * and 6.5×10^{-22}**, Fig 4.6) and not background due to random association of pixels. Animals with PrP^C switched off on FDCs had statistically significantly lower levels of PrP^C staining in comparison to non-Cre expressing spleens ($P< 1.0 \times 10^{-23}$ and 9.0×10^{-24}, Fig 4.6).

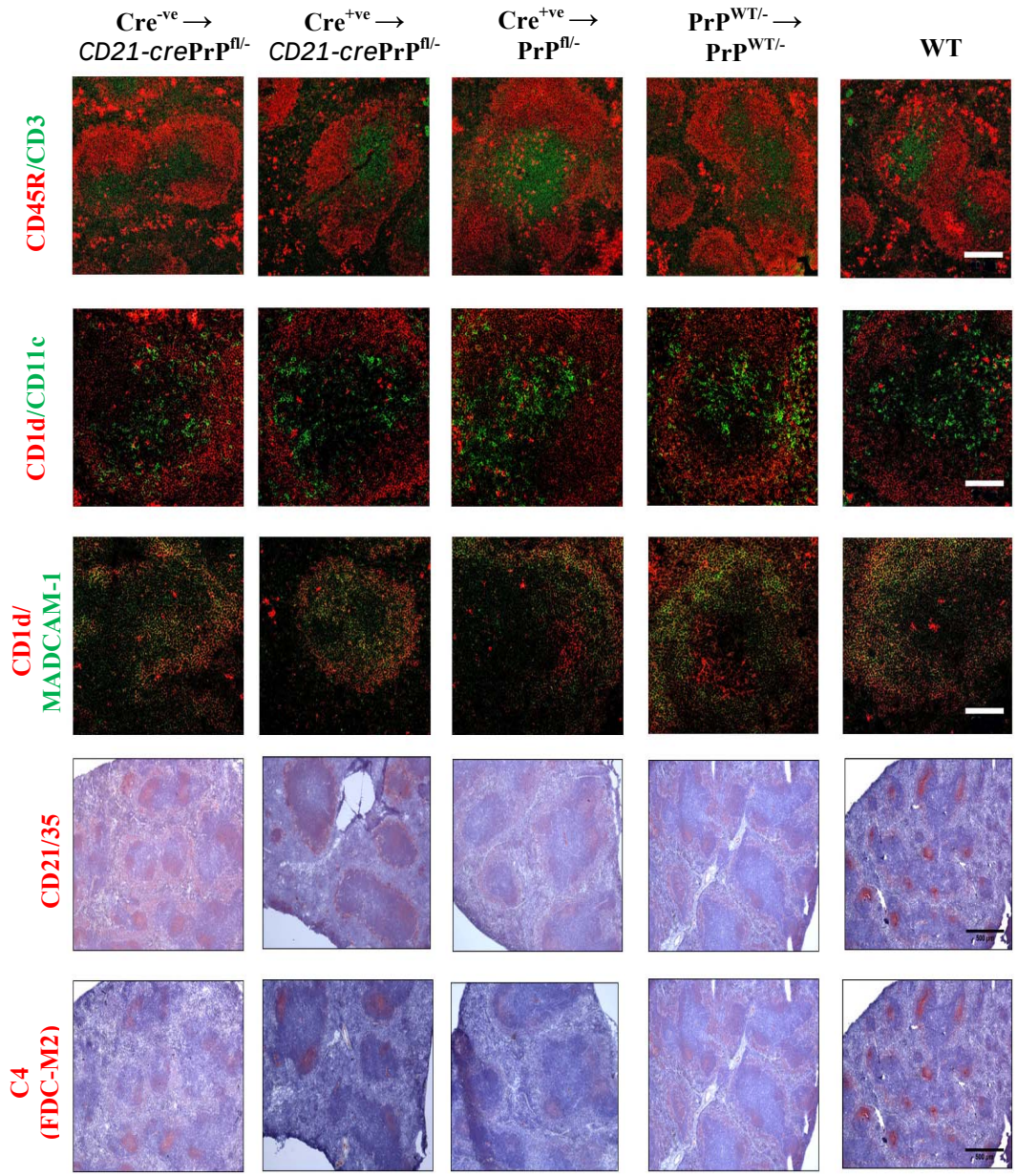
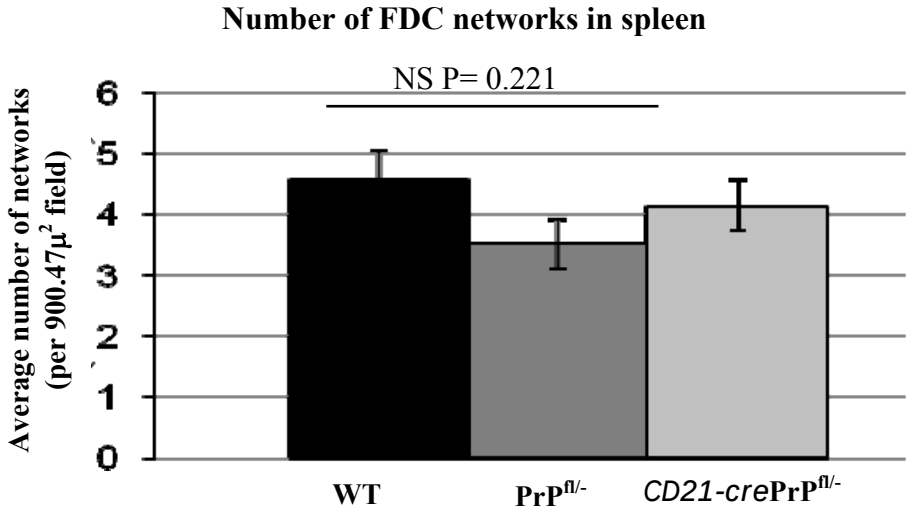


Figure 4.3 Insertion and expression of the transgenes and irradiation and bone marrow reconstitution has no effect on the microarchitecture of peripheral lymphoid tissues

Frozen spleen sections from all experimental transgenic mouse lines were immunolabelled for B lymphocyte subsets (CD45R and CD1d), T lymphocytes (CD3), FDCs (C4 and CD21/35), classical DCs (CD11c) and marginal zone cells (MADCAM-1 and CD1d). Comparison of sections from transgenic animals with WT controls showed no differences in the number or location of cell subsets within the spleen. Scale bar on flourescent images 100 μm. Scale bar on light microscopy images 500 μm and sections counterstained with haematoxylin, blue.

a.



b.

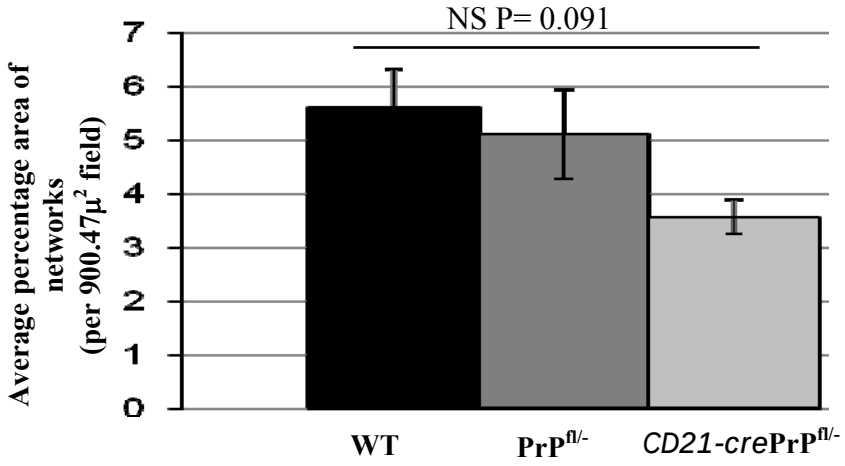


Fig 4.4 Expression of transgenes has no effect on the number or area of FDC networks in the spleen

Frozen spleen sections from WT, $PrP^{fl/-}$ and *CD21-cre*$PrP^{fl/-}$ animals were immunolabelled for FDCs using an anti CD35 monoclonal antibody. For each mouse line, 4 spleen sections 50 μm apart were immunolabelled and 4 images of 900.47μ^2 were taken per section.

a. FDC networks were manually counted for each image and the average number of FDC networks per image was calculated. No significant difference was observed in the number of FDC networks per 900.47μm^2 of spleen in transgenic animals in comparison to WT controls using a one-way anova (p=0.221)

b. The area of FDC networks per image was determined using ImageJ. This uses the total number of positive immunolabelled pixels per image to calculate a percentage area of a 900.47μm^2 of spleen positive for FDC network immunolabelling. No significant difference was observed in the percentage area of FDC immunolabelling in transgenic spleens in comparison to WT controls using a one-way anova (p=0.091)

4.3.5 Insertion and expression of transgenes and irradiation and bone marrow reconstitution have no effect on the distance between FDC networks and peripheral nerves in the spleen

Previous studies have shown that the distance between peripheral nerves and FDC networks can influence scrapie incubation period after peripheral inoculation (Glatzel, Heppner et al. 2001; Prinz, Heikenwalder et al. 2003). Therefore immunolabelling was used to further characterise the microarchitecture of lymphoid tissues of transgenic mice by measuring the distance between peripheral nerves and FDCs in the spleen. Frozen sections were immunolabelled for FDCs and peripheral nerves and LSM image browser software was used to measure the distance between them (Fig 4.7). Comparison of the average distance between FDCs and the nearest peripheral nerve showed there was no statistically significant difference in the distance between FDCs and peripheral nerves between transgenic spleens in comparison to WT counterparts (P=0.765, Fig4.7).

4.3.6 Insertion and expression of transgenes has no effect on immune complex trapping function of FDCs

A major function of FDCs in the immune system is to trap and retain antigen-containing immune complexes. In scrapie infection, removal of complement components C3 or C1q can significantly delay incubation period, suggesting that complement-mediated binding of scrapie agent to FDC networks is one possible method of scrapie agent localisation to the follicle (Cardone and Pocchiari 2001; Klein, Kaeser et al. 2001; Mabbott, Bruce et al. 2001; Mabbott 2004; Sim, Kishore et al. 2007). To ensure that FDCs in transgenic animals were not functionally

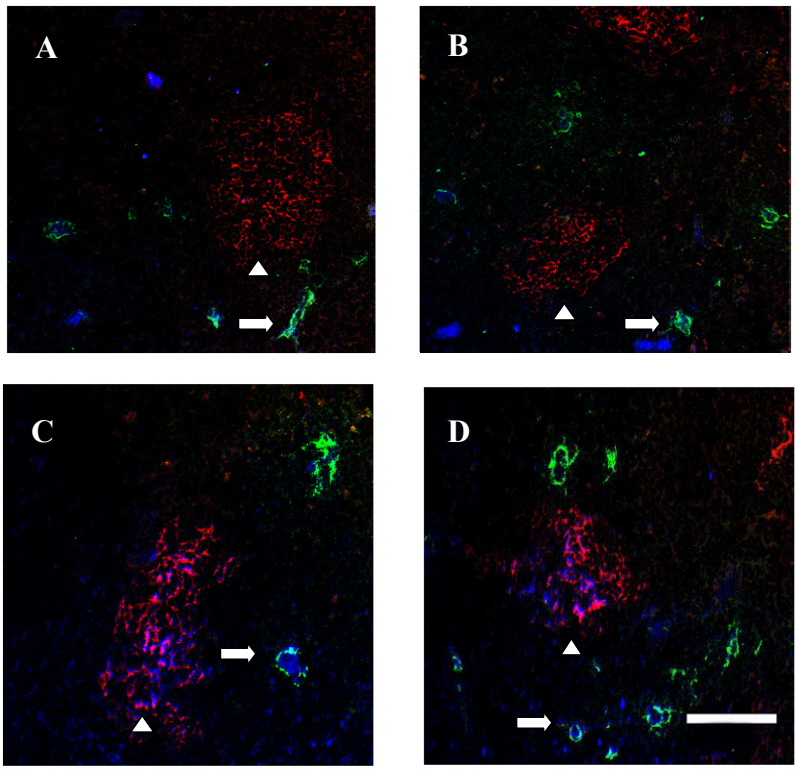


Fig 4.5 PrP^C expression is removed specifically on FDC networks in animals which express Cre

Immunolabelling of PrP^C (blue) on FDC networks △ (CD35/red) and peripheral nerves ⇨ (Tyrosine hydroxylase/green) in spleen sections from transgenic mouse lines. Animals were lethally γ-irradiated, received donor bone marrow 24 h later and were culled 100 days post reconstitution to allow replacement of all host-derived lymphocytes. Spleen sections from 6 animals from each line were labelled and analysed. *CD21-cre*$PrP^{fl/-}$ animals which received Cre^{-ve} (A) or Cre^{+ve} (B) bone marrow had no PrP^C labelling on their FDC networks however PrP^C labelling was detected on the peripheral nerves. These data demonstrate that expression of PrP^C has been removed specifically on the Cre-expressing FDCs.

$PrP^{fl/-}$ animals which received Cre^{+ve} bone marrow (C) and $PrP^{WT/-}$ animals which received $PrP^{WT/-}$ bone marrow (D) show PrP^C labelling on both FDC networks and peripheral nerves. Scale bar 100 μm.

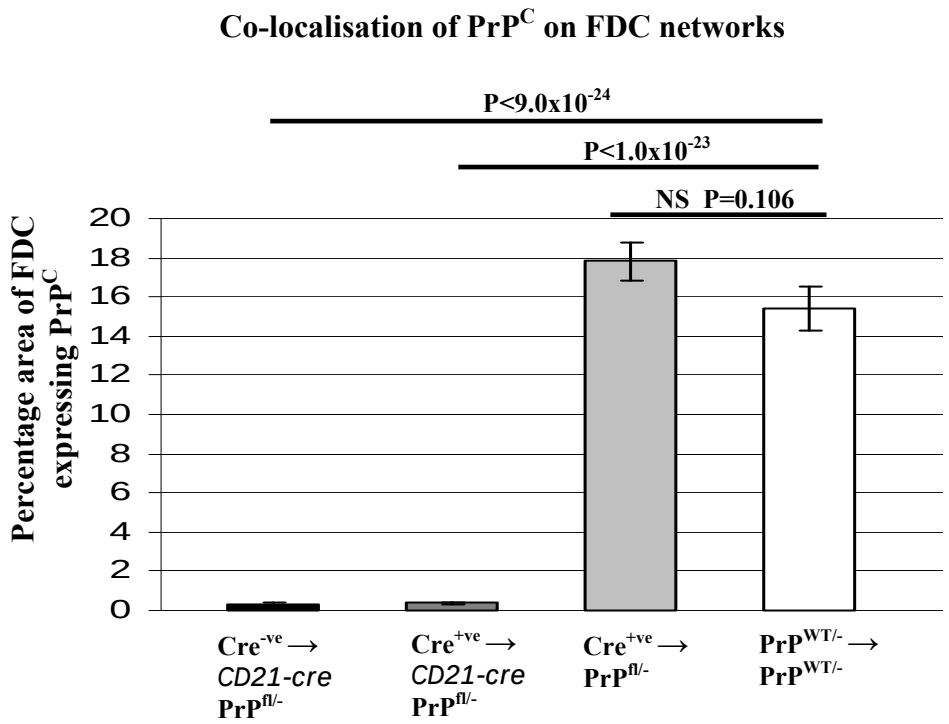


Fig 4.6 Expressionof Cre in FDCs under the *Cr2* promoter specifically removes PrP^C immunolabelling on FDC networks

Frozen spleen sections were immunolabelled for PrP^C, FDC and peripheral nerves. For each mouse line, spleens were taken from 6 animals. Two sections, 50μm apart, were immunolabelled for each spleen and 4 images per section were taken. This resulted in the analysis of 48 images for each mouse line using ImageJ image analysis software. For each image the number of pixels of each colour were counted using the multiple colour analysis macro allowing values to be obtained for total number of red (FDC) blue (PrP^C) and magenta (PrP^C co-localised with FDC) pixels per image. These values allowed the calculation of the average percentage of FDC area co-localised with PrP^C. In *CD21-cre*$PrP^{fl/-}$ animals, which have Cre-expressing FDCs this value is almost 0 and is significantly lower than $PrP^{fl/-}$ or $PrP^{WT/-}$ animals which do not have Cre-expressing FDCs. These data show that PrP^C has been switched off specifically on the Cre-expressing FDCs.

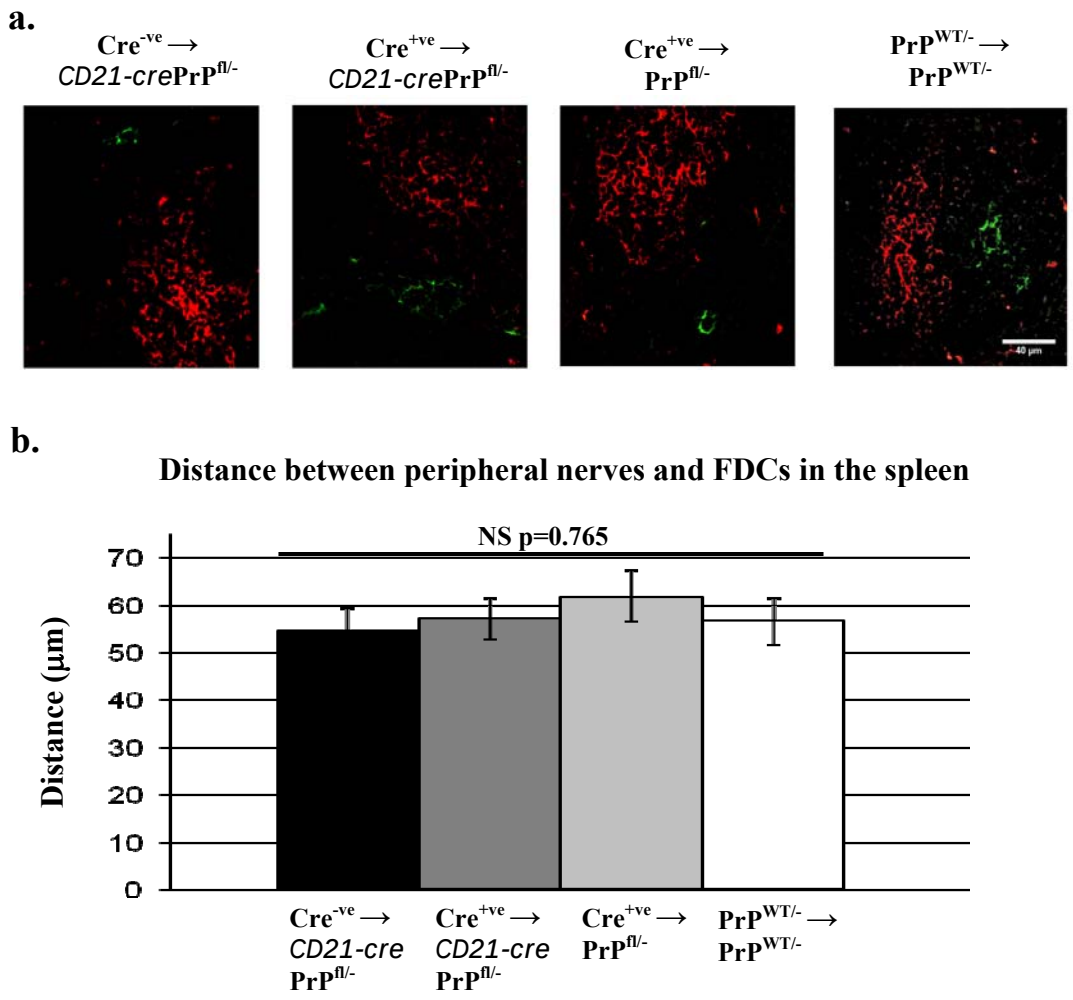


Fig 4.7 Insertion and expression of the transgenes has no effect on the distance between peripheral nerves and FDCs in the spleen

a. Frozen spleen sections from transgenic and WT lines were immunolabelled for FDC networks (CD35/red) and peripheral nerves (Tyrosine hydroxylase/green). For each mouse line, spleens were taken from 6 animals. Two sections, 50 µm apart, were immunolabelled for each spleen and 4 images per section were taken. This esulted in 48 images analysed for each mouse line. Scale bar 50 µm.

b. The distance between the FDC networks and peripheral nerves was measured using LSM image browser software (Zeiss). The average distance between nerves and FDCs was calculated for each mouse line. Analysis using a one-way anova test determined there was no significant difference in the distance between the FDC networks and peripheral nerves in the spleens of transgenic lines in comparison to WT controls (p=0.765).

impaired, immune complex trapping was assessed and compared with that of WT animals. Transgenic and WT control lines were injected with pre-formed rabbit PAP immune complexes. Animals were culled 24 hours later and spleens were harvested for analysis. Frozen sections were stained for rabbit immunoglobulin (Ig) and co-localisation of rabbit Ig on FDC networks was measured using ImageJ software as described previously. There were no observed differences in levels of immune complex trapping by FDCs between transgenic animals and WT controls (P=0.085, Fig 4.8). Uninjected control animals showed no immunolabelling of rabbit Igs on the FDC networks. These data show that insertion and expression of Cre under the *Cr2* promoter has no effect on FDC immune complex trapping function.

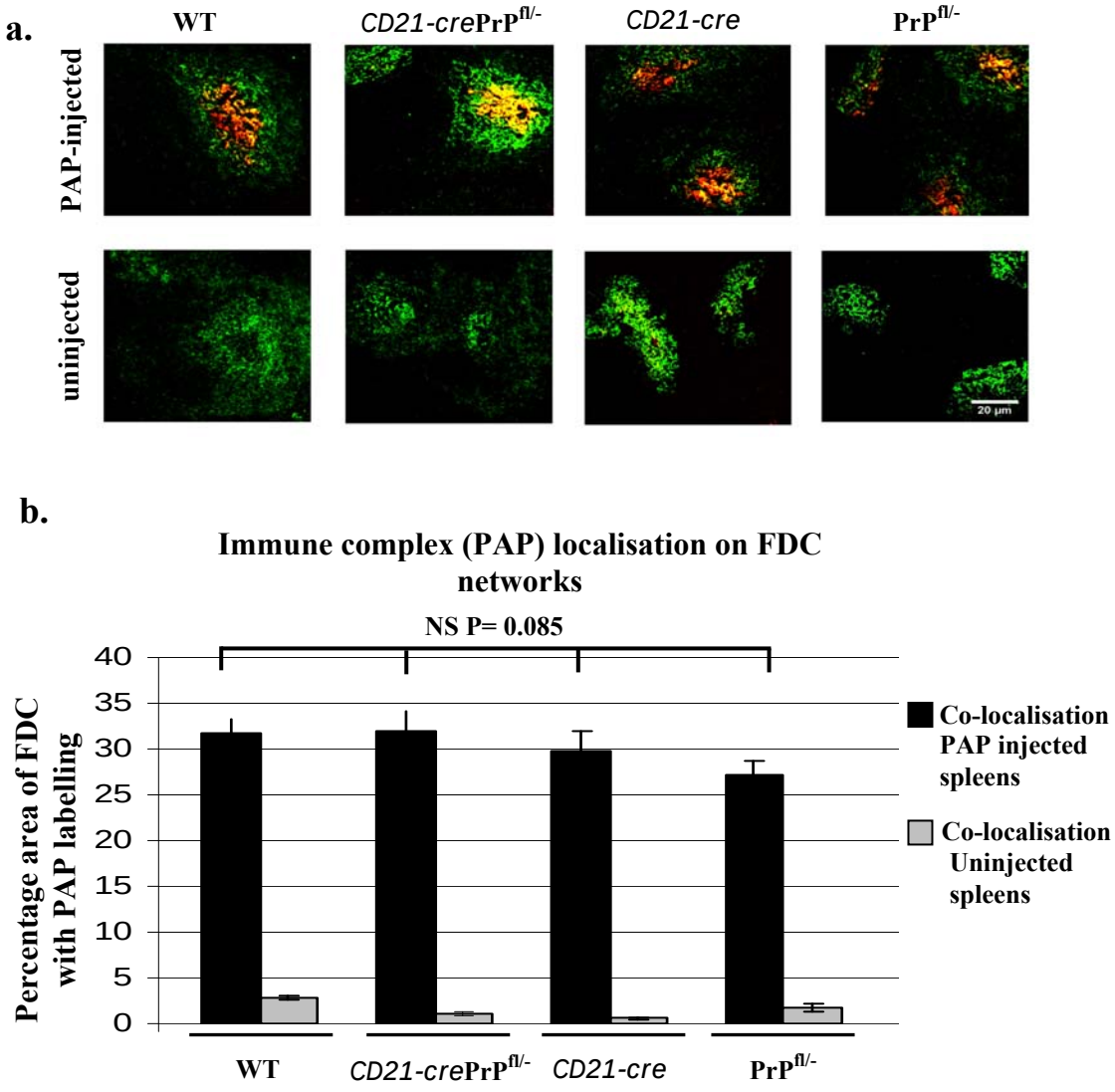


Figure 4.8 Insertion and expression of transgenes has no effect on immune complex trapping by FDCs

Transgenic and WT mice were injected with preformed rabbit peroxidise-anti-peroxidase (PAP) immune complexes (n=4). Spleens were harvested 24 h later for analysis. Uninjected controls for each line (n=2) were also collected.

a. Frozen sections were immunolabelled for FDCs (CD35/green) and rabbit Ig (red) to detect co-localisation of PAP on FDC networks. Original magnification x 200.

b. Images were analysed for co-localisation using an ImageJ macro described previously (Fig 4.6). For each animal, two spleen sections 50 µm apart were immunolabelled and 5 images were taken per section. A total of 40 images per mouse line for PAP-injected, and 20 images per mouse line for uninjected controls, were analysed for co-localisation. There is no significant difference in the percentage area of FDC co-localised with PAP between WT and transgenic lines. From this data, it can be concluded that insertion and expression of the transgenes has no functional effect on FDC immune complex trapping.

4.4 Discussion

The *CD21-cre* line was crossed with a $PrP^{fl/fl}$ line to create a model where PrP^{C} could be switched off exclusively on FDCs. In this chapter the *CD21-cre*PrP^{fl} line was extensively characterised to determine if it would be an efficient tool to study the role of FDCs after peripheral scrapie exposure. The characterisation studies carried out on this line indicate that PrP^{C} was switched off specifically on Cre-expressing FDCs and remained present on other non-Cre-expressing cells within the lymphoid tissue such as the nerves. Furthermore, insertion and expression of the transgenes had no additional effects on the lymphoid tissue that could influence scrapie pathogenesis.

The first breeding strategy used to create this line with homozygous $PrP^{fl/fl}$ resulted in the *Cr2* promoter losing control of Cre expression and all floxed *Prnp* DNA within the animal was recombined, resulting in a PrP deficient animal. As multiple generations of mice were produced containing both the *Cre* and the floxed *Prnp* transgenes, it is possible that Cre was expressed in the germline cells. If this occurs and both the *Cre* and *loxP* transgenes are inherited from one parent, this can result in Cre being expressed in all tissues and complete recombination of the floxed DNA throughout the animal (Schmidt-Supprian and Rajewsky 2007). To compensate for this, the *CD21-cre* line was crossed with a $PrP^{-/-}$ line and resulting *CD21-cre*$PrP^{-/-}$ progeny were crossed in one generation with the $PrP^{fl/fl}$ line to create *CD21-cre*$PrP^{fl/-}$ line. By eliminating generations of mice which had both the *Cre* and *loxP* transgenes present, the problem of Cre-mediated DNA recombination in the entire animal was also eliminated. Therefore the *CD21-cre*$PrP^{fl/-}$ line was used in subsequent characterisation and scrapie infection experiments.

PrP^C expression was successfully switched off in the FDCs of Cre-expressing animals. PCR analysis of DNA extracted from spleens confirmed the presence of the *Cre* transgene and recombination of the $Prnp^{fl}$ transgenes. Immunolabelling of spleen sections confirmed that PrP^C was removed on FDCs but present on non-CD21 expressing cells such as peripheral nerves, leaving little/no PrP^C detectable by immunolabelling. This is in agreement with previous studies using the CD21-cre line, which measured 96% of floxed DNA was recombined in CD21-cre mouse line crosses (Victoratos, Lagnel et al. 2006).

Characterisation of the lymphoid tissues of transgenic experimental lines was essential to ensure that any differences in scrapie pathogenesis were due to removal of PrP^C on the FDC and not due to changes in the lymphoid tissues caused by the insertion of the transgenes. Depletion of $CD11c^+$ cells and B lymphocytes has been shown to delay the onset of peripherally acquired scrapie (Klein, Frigg et al. 1997; Raymond, Aucouturier et al. 2007). Therefore, various cell subsets were immunolabelled on spleen sections from transgenic experimental and WT lines to determine if there were any differences in michroarchitecture caused by insertion and expression of the transgenes. No observable difference could be seen in the number or location of B lymphocytes, T lymphocytes, dendritic cells or marginal zone cells in the spleen.

The presence of mature FDC networks in the spleen is also essential for efficient scrapie pathogenesis and changes in the number or cellular location of FDCs can alter scrapie pathogenesis (Brown, Stewart et al. 1999; Mabbott, Williams et al. 2000a; Mabbott, McGovern et al. 2002; Mabbott, Young et al. 2003; Prinz, Heikenwalder et

al. 2003). Analysis of FDC networks in the spleens of transgenic mice showed there were no differences in the number and area of FDC networks in comparison to WT controls. The function of the FDC networks in transgenic mice was also analysed by measuring their ability to trap pre-formed immune complexes. No measureable differences in the level of immune complex trapping on FDCs of transgenic lines in comparison to WT controls was detected indicating that insertion and expression of the transgenes had no effect on FDC function. The distance between peripheral nerves and FDC networks can also influence scrapie incubation period (Glatzel, Heppner et al. 2001; Prinz, Heikenwalder et al. 2003). This was measured in spleen sections from transgenic experimental lines and WT controls and no significant difference was measured in the distance between the FDC networks and nerves.

Taken together, these data show that the insertion and expression of the transgenes in experimental mice has no effects on the microarchitecture of the spleen or the number or distribution of various cell subsets found within. These data confirm previous studies using the *CD21-cre* mice which did not report any toxic effects caused by the *Cre* transgene and showed no differences in FDC and B lymphocyte numbers or location in the spleen (Kraus, Alimzhanov et al. 2004; Victoratos, Lagnel et al. 2006; Schenten, Kracker et al. 2009). Additionally, characterisation of the $PrP^{-/-}$ line has shown no overt phenotype even though PrP^{C} is ubiquitously expressed (Manson, Clarke et al. 1994a).

Characterisation of the CD21-cre$PrP^{fl/-}$ mouse line has shown that PrP^{C} is efficiently removed in Cre-expressing FDCs but remains present on other cell types within the lymphoid tissues. Furthermore, insertion and expression of the transgenes have no

additional effects on the cellular composition or structure of the lymphoid tissue. Therefore the CD21-cre$PrP^{fl/-}$ mouse line is an effective model to study the role of FDCs after peripheral exposure to scrapie. This line will be used to determine if PrP^{C} expressing FDCs are required for efficient scrapie agent replication in the spleen (Chapter 5).

CHAPTER 5

Effect of FDC-restricted PrP ablation on TSE agent accumulation in the spleen

5.1 Abstract

After peripheral exposure to TSE agents, PrP^{Sc} and infectivity usually accumulates on FDCs in the lymphoid tissues. Models used so far have not been able to definitively distinguish the role of the FDCs from that of all other stromal, lymphoid and neural cells found within the lymphoid tissue. For this reason, it is possible that rather than actively replicating the scrapie agent, FDCs may simply accumulate the agent after replication on another cell type within the lymphoid tissue. Characterisation of the *CD21-cre*$PrP^{fl/-}$ model (Chapter 4) confirmed that *CD21-cre*$PrP^{fl/-}$ mice reconstituted with WT bone marrow switch off PrP^{C} expression exclusively on FDCs. These mice were inoculated ip with the ME7 scrapie agent to determine if FDCs simply acquire the TSE agent on their surface.

After initial localisation of the scrapie agent to the FDC network, animals with Cre-expressing, PrP^{C} deficient FDCs were unable to replicate the scrapie agent in the lymphoid tissue when inoculatied ip with ME7 scrapie. In the absence of replication, PrP^{Sc} was scavenged from the FDC network by TBMs and possibly degraded. Furthermore, animals with Cre-expressing, PrP^{C} deficient FDCs inoculated ic with ME7 scrapie were susceptible to scrapie infection and had positive neuropathology and PrP^{Sc} deposition in their brains. However, spleens from these animals remained free from PrP^{Sc} accumulation. In conclusion, these data show that PrP^{C}-expressing FDCs actively replicate the TSE agent in the periphery prior to neuroinvasion and do not simply accumulate infectivity on their surface after replication on another cell type.

5.2 Introduction

PrP^{C} expression by FDCs is considered essential for effective scrapie pathogenesis after peripheral exposure. Furthermore, heavy accumulations of TSE-agent-specific disease-associated PrP^{Sc} occur upon the FDC networks in lymphoid tissues of scrapie-affected mice (McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Jeffrey, McGovern et al. 2000). Previous data suggest that FDCs have a role in replicating the scrapie agent within the lymphoid tissue, however as of yet, there has not been a suitable model which has been able to exclusively address this issue (Brown, Stewart et al. 1999; Montrasio 2000; Mabbott, Williams et al. 2000a; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Mohan, Bruce et al. 2005). As the main function of FDCs within the B lymphocyte follicles is to pick up and retain antigen-containing immune complexes on their surfaces, the possibility remains that FDCs are simply acquiring the scrapie agent after replication on another lymphoid, stromal or neural cell types within the lymphoid tissues. Furthermore, FDCs have been shown to acquire expression of proteins that they themselves do not express, such as class II MHC, via exosomes (Gray, Kosco et al. 1991; Thery, Regnault et al. 1999; Denzer, van Eijk et al. 2000).

To definitively determine the role of FDCs in scrapie pathogenesis, the *CD21-cre* mouse line was crossed with the $Prnp^{fl}$ mouse line to create a model in which PrP^{C} expression was switched off exclusively on CD21-expressing cells. These animals were subsequently irradiated and reconstituted with non-*Cre*-expressing bone marrow to restrict the removal of PrP^{C} expression exclusively to the FDC network. Characterisation of this compound transgenic model (Chapter 4) showed that PrP^{C}

expression was efficiently and specifically removed only on the FDC networks of *CD21-cre*PrP$^{fl/-}$ mice. Furthermore, no adverse effects on the status or function of FDC networks, or in the general michroarcitecture of the lymphoid tissue were observed. These data confirmed that the *CD21-cre*PrP$^{fl/-}$ model would be a useful tool to determine the role of PrP^{C}-expressing FDCs in scrapie pathogenesis and elucidate whether FDCs simply accumulate TSE agent on their surfaces after replication on another cell type. As host expression of PrP^{C} is essential for TSE agent pathogenesis (Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997), if FDCs themselves actively replicate the TSE agent, then efficient pathogenesis of TSE agent in peripheral lymphoid tissues should not be possible when PrP^{C} expression is removed exclusively on the FDC network. If however FDCs simply acquire the TSE agents, then in the absence of PrP^{C} expression, heavy accumulation of PrP^{Sc} would still be detected.

*CD21-cre*PrP$^{fl/-}$ mice were inoculated ip with ME7 scrapie and culled at 35 or 70 dpi. Further animals were also inoculated ic with ME7 scrapie to ensure that transgenic lines were susceptible to TSE infection. Spleens were harvested and detection of PrP^{Sc} was used to assess the pathology of scrapie. PrP^{Sc} has been shown to co-purify with TSE agent infectivity and is considered by many to be the sole component of the TSE infectious agent (Bolton, McKinley et al. 1982).

Experiments demonstrated that in animals with PrP ablation specifically in FDCs, the scrapie agent, as detected by PrP^{Sc} immunolabelling, initially located to the FDC network in the follicle. However at later time points PrP^{Sc} was found only within the TBMs. This is in contrast to control animals which retained *Prmp*$^{+/-}$ FDCs, where

PrP^{Sc} located to the FDC network in the follicle and accumulation increased over time suggesting replication of the scrapie agent. These data confirm that PrP^{C}-expressing FDCs actively replicate the TSE agent. When PrP^{C} is ablated specifically on FDCs, no accumulation of PrP^{Sc} occurs on the FDC surface, demonstrating that FDCs do not acquire the scrapie agent after replication on another cell type. Instead, TBMs in the follicle clear PrP^{Sc} from the FDC network and possibly degrade it.

5.3 Results

5.3.1 Experimental design

To determine the role of FDCs in TSE agent replication, the *CD21-cre*$PrP^{fl/-}$ mouse line which has PrP^{C} switched off specifically on FDCs was inoculated ip with ME7 scrapie. Animals were aged to 8 weeks, lethally γ-irradiated and given donor bone marrow 24 h later. To restrict the PrP ablation specifically to FDCs, *CD21-cre*$PrP^{fl/-}$ mice were given bone marrow from Cre-negative littermates. However other transgenic host/bone marrow combinations were also produced as control lines and these are summarised in Table 5.1. Animals were used in subsequent experiments at 100 d post bone-marrow reconstitution to allow for efficient replacement of host bone-marrow derived cells with donor counterparts. Mice from each group were inoculated with 20 μl of a 1% (wt/vol) scrapie brain homogenate. Animals were then culled at 35 and 70 days post-inoculation (dpi) and tissues were collected to assess the cellular sites of PrP^{Sc} accumulation in the spleen. A summary of experimental design can be found in Figure 5.1.

Cellular sites of PrP^{Sc} accumulation in the spleen were determined by immunolabelling of PrP. Current antibodies used to detect PrP cannot distinguish between the normal cellular isoform of the prion protein, PrP^{C}, and the scrapie-associated, relatively PK-resistant isoform, PrP^{Sc}. For this reason, the disease-specific PrP detected by immunohistochemistry in spleens of infected animals is termed PrP^{d}. All immunohistochemistry was carried out using the rabbit anti-PrP polyclonal antibody (PAb) 1B3 and confirmed using the mouse anti-PrP monoclonal (MAb)

Host Genotype	Donor Genotype	PrP^C switched off	Nomenclature
CD21-cre$PrP^{fl/-}$	$PrP^{fl/-}$	FDCs only	Cre^{-ve} → *CD21-cre*$PrP^{fl/-}$
CD21-cre$PrP^{fl/-}$	*CD21-cre*$PrP^{fl/-}$	FDCs and B lymphocytes	Cre^{+ve} → *CD21-cre*$PrP^{fl/-}$
$PrP^{fl/-}$	*CD21-cre*$PrP^{fl/-}$	B lymphocytes only	Cre^{+ve} → $PrP^{fl/-}$
$PrP^{WT/-}$	$PrP^{WT/-}$		$PrP^{WT/-}$ → $PrP^{WT/-}$

Table 5.1 Summary of experimental lines used in scrapie experiments

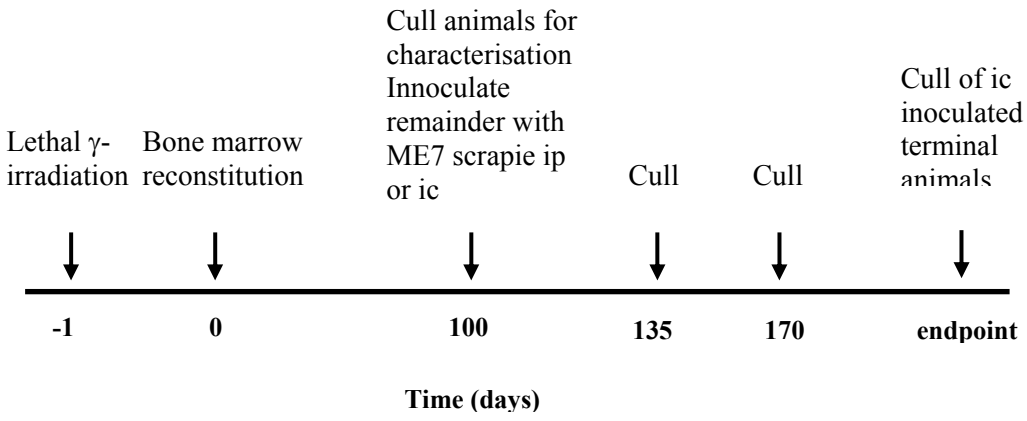


Figure 5.1 Summary of experimental design

antibody 6H4. As both antibodies gave similar results, only immunohistochemistry images from 1B3 labelling are shown.

Treatment with PK digests the cellular PrP^{C} leaving PK-resistant core of PrP^{Sc} intact (Manson, Clarke et al. 1994b). PrP^{C} and PrP^{Sc} can be distinguished on histological sections using the PET blot method**.** This method uses PK treatment of tissue sections on nitrocellulose membrane prior to immunolabelling with anti-PrP antibodies, to destroy PrP^{C}. Therefore any PrP detected by this method can be confirmed as the scrapie-associated PrP^{Sc}. Detection of PrP^{d} and PrP^{Sc} have been confirmed by many studies to be reliable markers of TSE disease and in most cases correlate closely with presence of TSE agent infectivity (Bolton, McKinley et al. 1982; McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009).

5.3.2 Effect of FDC-restricted PrP ablation on TSE agent accumulation in the spleen at 35 days post-exposure

Animals were culled at 35 dpi with the ME7 scrapie agent and spleens were harvested for analysis of the cellular sites of PrP^{Sc} accumulation. In all spleens with Cre-deficient, PrP^{C} expressing FDCs, accumulation of the abnormal, disease-associated PrP^{d} occurred in the germinal centres of the spleen (Figs 5.2). This PrP^{d} was located to the FDC network as shown by immunolabelling of serial sections for PrP^{d}, FDCs and B lymphocytes (Fig 5.3). However, as animals are only expressing half copy

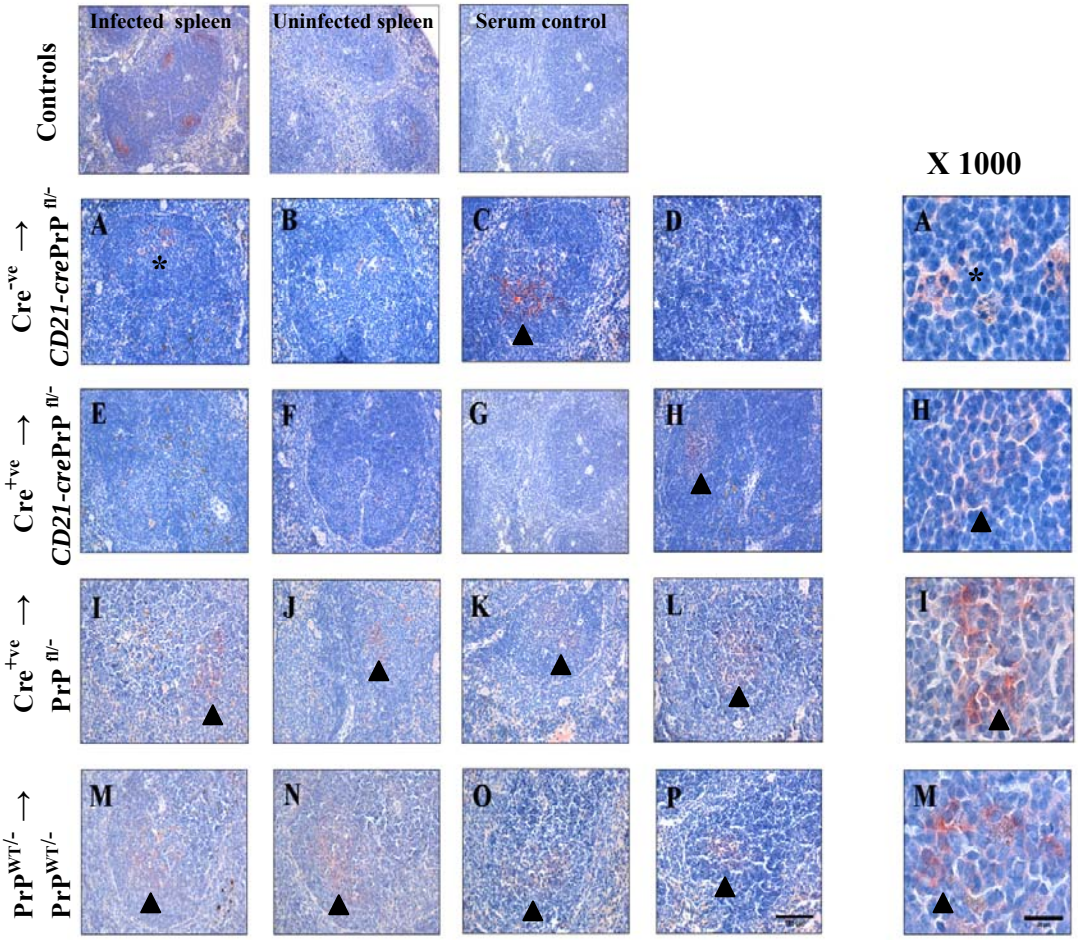


Figure 5.2 PrP^d immunolabelling in the spleen at 35 dpi with the ME7 scrapie agent

Four animals from each experimental line were culled at 35 dpi with ME7 scrapie and spleens were immunolabelled to detect PrP^d (using PAb 1B3, red).

All spleens with Cre-deficient, PrP^C-expressing FDCs (I-P) show PrP^d immunolabelling at low levels on the FDC networks (▲) and tingible body macrophages (TBMs, *) in the follicles. Spleens from some animals with Cre-expressing, *Prnp*$^{-/-}$ FDCs (A-H) also show immunolabelling on the FDC network, however this is detected at a lower frequency with only 2/8 animals (C, H) with positive PrP^d immunolabelling in the FDC networks. Some of these animals instead show PrPd labelling located only within the TBMs of the follicle (A). Scale bar on main figure100 μm. Scale bar on x 1000 images 20μm. Sections counterstained with haematoxylin, blue.

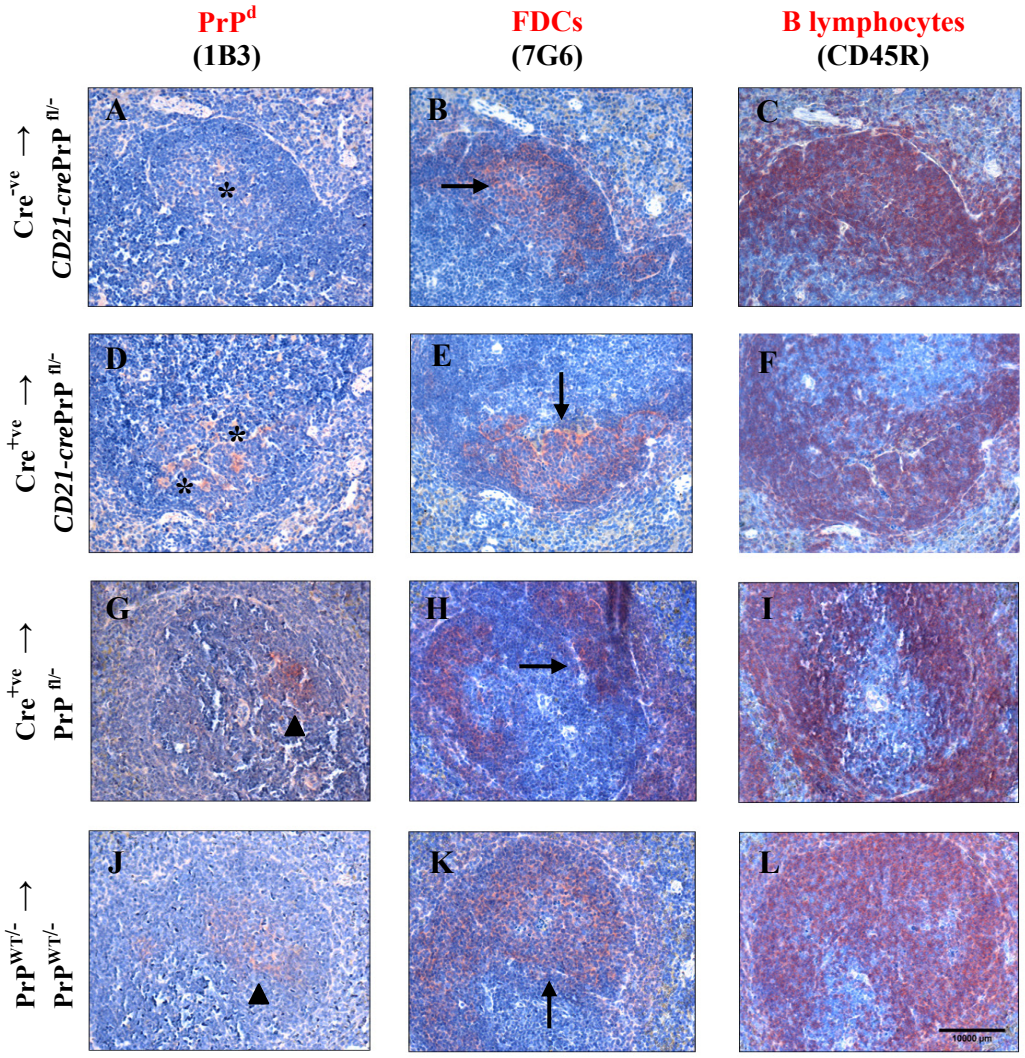


Fig 5.3 Immunohistochemical analysis of PrP, FDC and B lymphocytes in spleens of mice taken at 35 dpi after exposure to the ME7 scrapie agent

Serial sections from spleens were immunolabelled for PrP (PAb 1B3, red), FDCs and $CD21^+$ B lymphocytes (MAb 7G6, red) and B lymphocytes (MAb CD45R, red) to determine if PrP^d detected co-localised to the FDC network. One animal is shown from each experimental transgenic line.

In spleens with Cre-deficient, PrP^C-expressing FDCs (G-L), PrP^d (▲) is localised to the FDC networks (→) as shown in serial sections. PrP^d location in spleens with Cre-expressing, PrP^C deficient FDCs (A-F) was variable at 35 dpi. PrP^d was present on the FDCs of some follicles but in others was found exclusively within TBMs (*) in the B lymphocyte follicles. Scale bar 100 µm. Sections counterstained with haematoxylin, blue.

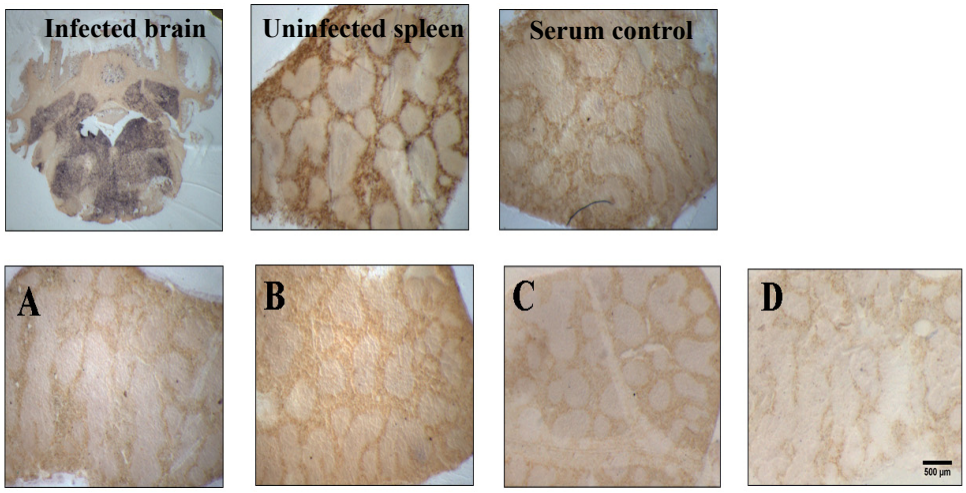


Figure 5.4 PET blot analysis of PrP^{Sc} accumulation in spleens taken at 35 dpi with the ME7 scrapie agent
Paraffin-embedded sections on nitrocellulose membrane were treated with PK to remove any native PrP^{C}, then immunolabelled (PAb 1B3, blue/black) to detect any remaining PrP^{Sc}. One representative example from each transgenic line is shown. **A,** Cre^{-ve}→*CD21-cre*$PrP^{fl/-}$; **B,** Cre^{+ve}→*CD21-cre*$PrP^{fl/-}$; **C,** Cre^{+ve}→$PrP^{fl/-}$; **D,** $PrP^{fl/-}$→$PrP^{fl/-}$. Scale bar 500 µm.

Due to the low levels of PrP^{d} detected in infected tissue at this stage by immunohistochemistry (Fig 5.2), PrP^{Sc} is not readily detected after PK treatment in any of the transgenic experimental lines. Therefore it is not confirmed that the PrP^{d} detected by immunohistochemistry in these tissues is PrP^{Sc} at this timepoint.

number levels of *Prnp* and were culled early in the incubation period, the magnitude of PrP^{d} accumulation was insufficient to be confirmed as PrP^{Sc} by the PET blot method (Fig 5.4). Therefore it could not be confirmed that PrP^{d} detected by immunohistochemistry was indeed scrapie-associated PrP^{Sc}.

In animals with Cre-expressing, PrP^{C}-deficient FDCs, spleen sections taken at 35 dpi showed variable deposition of PrP^{d} in the follicles. Only 3 out of 8 animals had detectable PrP^{d} by immunohistochemistry (Figs 5.2). This deposition was located to the FDCs in some animals, but only found within TBMs within the follicle in others (Fig 5.3). As with animals with PrP^{C}-expressing FDCs, levels of PrP^{d} accumulation were too low to be confirmed by the PET blot method as PrP^{Sc} (Fig 5.4).

5.3.3 Effect of FDC-restricted PrP-ablation in TSE agent accumulation in the spleen 70 days post-exposure

Animals were culled at 70 dpi with ME7 scrapie and spleens were harvested for analysis of the cellular sites of PrP^{Sc} accumulation. In all spleens with Cre-deficient, PrP^{C}-expressing FDCs, accumulation of the abnormal, disease-associated PrP^{d} occurred in the germinal centres of the spleen at a greater intensity than that seen in spleens from animals culled at 35 dpi (Figs 5.5). This PrP^{d} was located on the FDC network as shown by immunolabelling of serial sections for FDCs and B lymphocytes (Fig 5.6). Furthermore, the PrP^{d} accumulation detected upon the FDCs was confirmed to be PK-resistant, scrapie agent-associated PrP^{Sc} using the PET blot method (Fig 5.7). From these data it can be concluded that high levels of the scrapie

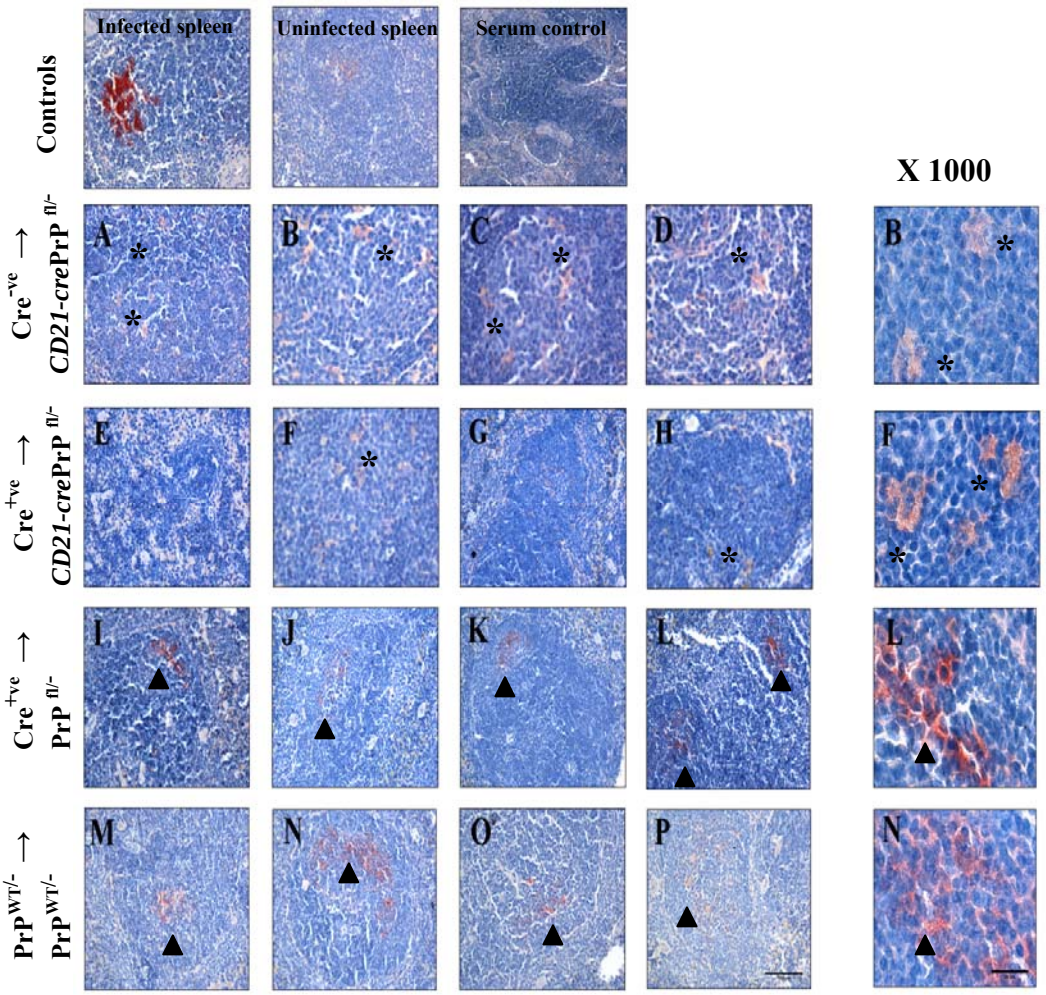


Figure 5.5 PrP^d immunolabelling in the spleen at 70 dpi with the ME7 scrapie agent

Four animals from each experimental line were culled at 70 dpi with ME7 scrapie and spleens were immunolabelled to detect PrP^d (PAb 1B3, red)

All spleens with Cre-deficient, PrP^C-expressing FDCs (I-P) show PrP^d immunolabelling on the FDC networks (▲) within the follicles at an increased level than that seen in spleens from animals culled at 35 dpi. Spleens from animals with Cre-expressing, PrP^C deficient FDCs (A-H) no longer show any labelling on the FDC networks as was detected in spleens from D35 animals. Instead, PrP^d is detected only within TBMs (*) in the follicle. This suggests the scrapie agent cannot replicate on PrP^C deficient FDCs and any PrP^{Sc} from innocula has been cleared from FDC networks and sequestered by TBMs in the follicle. Scale bar on main figure100 μm. Scale bar on x 1000 magnification images 20μm. Sections counterstained with haematoxylin, blue.

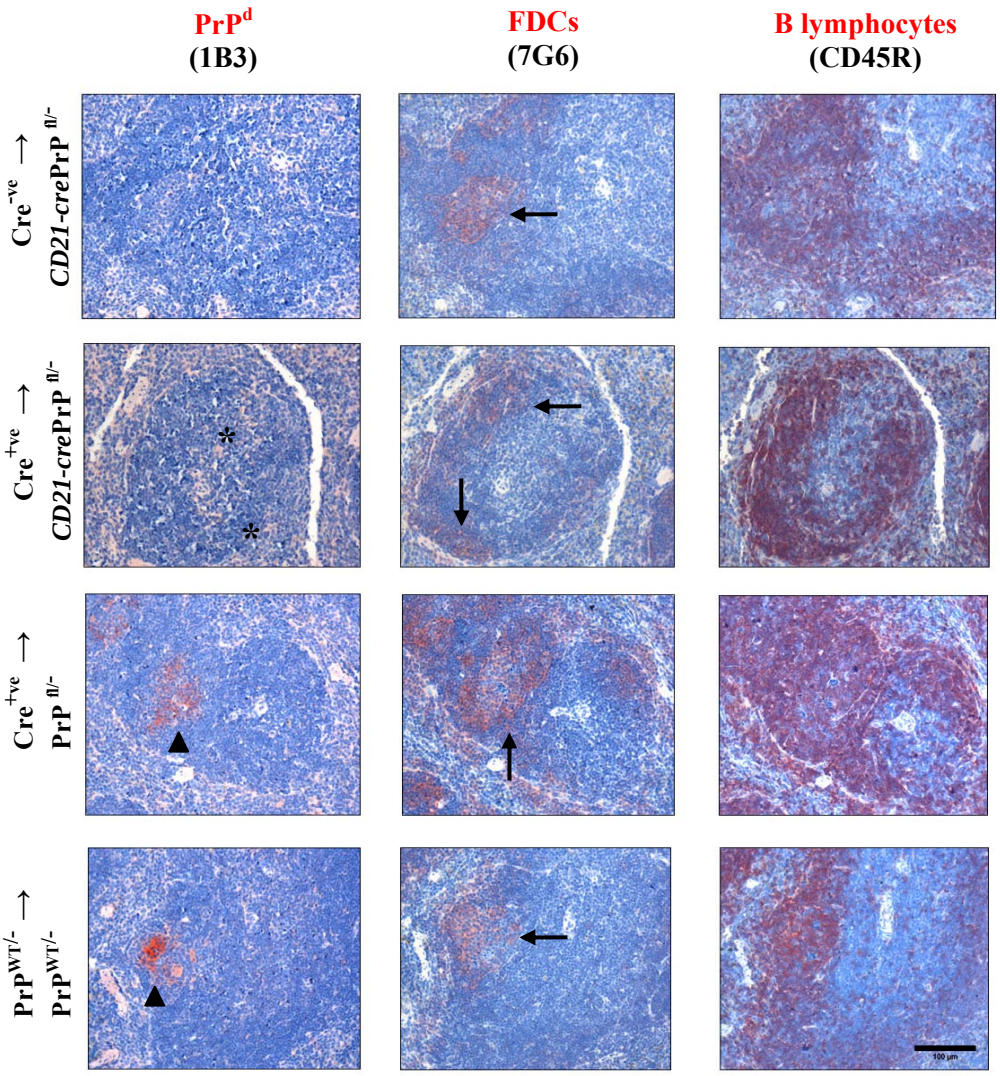


Fig 5.6 Immunohistochemical analysis of PrP, FDC and B lymphocytes in spleens of mice taken at 70 dpi with the ME7 scrapie agent

Serial sections from spleens were immunolabelled for PrP (PAb 1B3, red), FDCs and $CD21^+$ B lymphocytes (MAb 7G6, red) and B lymphocytes (MAb CD45R, red) to determine whether the PrP^d detected was co-localised to the FDC network. One animal is shown from each experimental transgenic line.

In spleens with Cre-deficient, PrP^C-expressing FDCs (G-L), PrP^d (▲) is localised to the FDC networks (⟶) as shown in serial sections. PrP^d location in spleens with Cre-expressing, PrP^C deficient FDCs (A-F) was found exclusively within TBMs (*) and didn't co-localise with FDC networks in the follicle. Scale bar 100 µm. Sections counterstained with haematoxylin, blue.

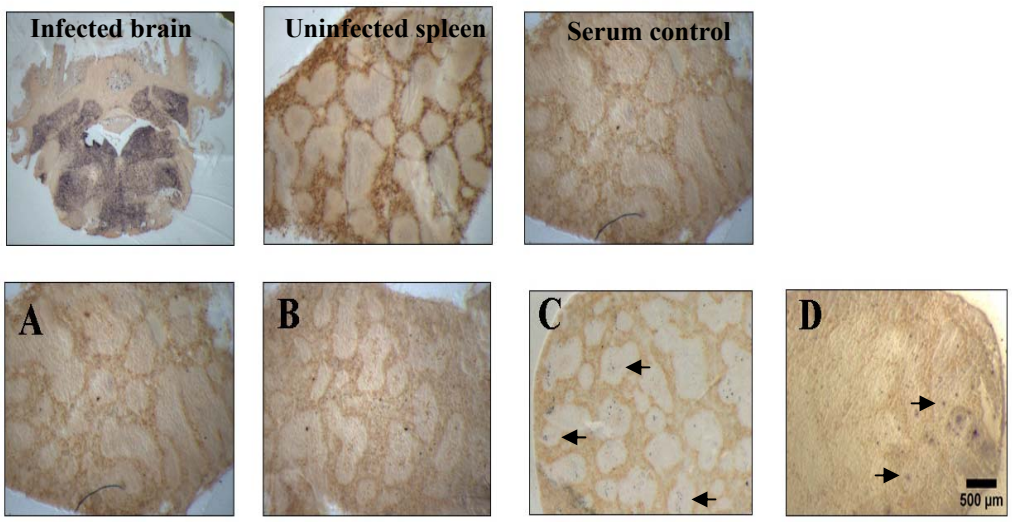


Figure 5.7 PET blot analysis of PrP^{Sc} accumulation in spleens taken at 70 dpi with the ME7 scrapie agent

Paraffin-embedded sections on nitrocellulose membrane were treated with PK to remove any native PrP^{C}, then immunolabelled (PAb 1B3, blue/black) to detect any remaining PrP^{Sc}. One representative example from each transgenic line is shown **A,** Cre^{-ve}→ *CD21-cre*$PrP^{fl/-}$; **B,** Cre^{+ve}→ *CD21-cre*$PrP^{fl/-}$; **C,** Cre^{+ve}→ $PrP^{fl/-}$; **D,** $PrP^{fl/-}$ → $PrP^{fl/-}$. Scale bar 500 µm

In spleens with Cre-deficient, PrP^{C}-expressing FDCs (C and D), some PrP labelling (➔) can be detected in the follicles after treatment with PK and can therefore be confirmed as the disease-associated PrP^{Sc}. In spleens with Cre-expressing, PrP^{C}-deficient FDCs (A and B), immunohistochemistry had shown PrP^{d} was present only within the TBMs (Fig 5.5). At the low levels of PrP^{d} observed within the TBMs, PrP^{d} is not readily detected after PK treatments. Therefore it is not confirmed that the PrP^{d} detected in these tissues by immunohistochemistry is PrP^{Sc}.

agent are able to accumulate on PrP^{C}-expressing FDCs as expected. In addition, as no significant differences are seen between levels of PrP^{Sc} in Cre^{+ve}→ $PrP^{fl/-}$ spleens and $PrP^{WT/-}$→ $PrP^{WT/-}$ spleens, it can be concluded that switching off PrP^{C} specifically on $CD21^{+}$ B lymphocytes in Cre^{+ve}→ $PrP^{fl/-}$ mice has no detectable impact on TSE agent accumulation upon the FDCs.

In animals with Cre-expressing, PrP^{C} deficient FDCs, spleen sections at 70 dpi no longer showed deposition of PrP^{d} on the FDC network. Instead if PrP^{d} was detected at all, it was only found within TBMs (Figs 5.5). Indeed, immunolabelling of serial sections for FDCs and B lymphocytes confirmed that the PrP^{d} immunolabelling did not co-localise with FDC networks (Fig 5.6). The, levels of PrP^{d} observed within the TBMs were too low to be confirmed by the PET blot method as PrP^{Sc} (Fig 5.7). From these data it can be concluded that PrP^{C}-expressing FDCs are essential for the scrapie agent to replicate within the lymphoid tissues. Without active replication of the agent by PrP^{C}-expressing FDCs, the scrapie agent appeared to be scavenged by TBMs in the follicle and possibly degraded. This suggests that PrP^{C}-expressing FDCs actively replicate the TSE agent and do not simply acquire it from other PrP^{C}-expressing cells present in the lymphoid tissue.

5.3.4 FDC-restricted PrP^{C} ablation has no effect on TSE disease when infection is established directly in the CNS

Some transgenic animals with Cre-expressing, PrP^{C} deficient FDCs were inoculated with the ME7 scrapie agent directly into the brain by ic injection to determine whether the nervous system in these animals was still susceptible to TSE disease. 129/Ola WT

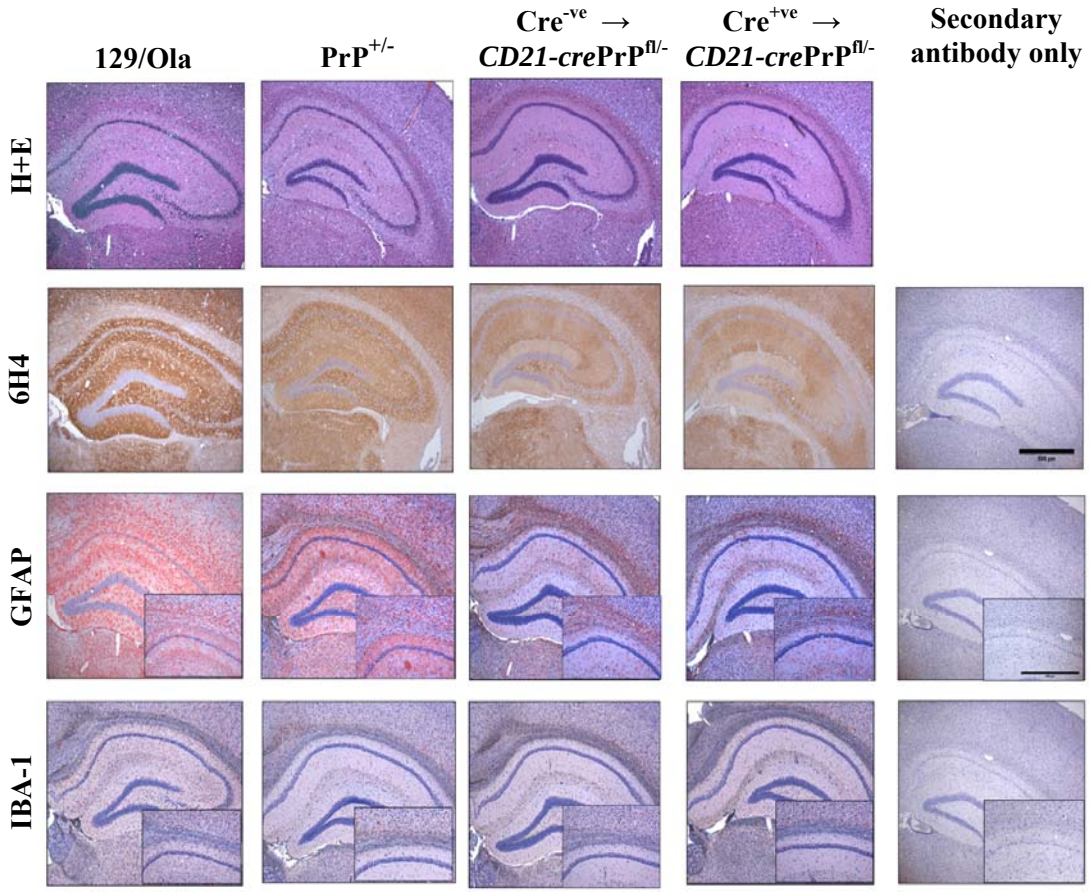


Fig 5.8 Neuropathology within the brains of ic injected, clinically scrapie-affected mice

Serial brain sections were immunolabelled to compare the characteristic neuropathological hallmarks of TSE disease: spongiform change (vacuolation, H+E), deposition of the abnormal disease associated PrP^{Sc} (PAb 6H4, brown) and proliferation and activation of astrocytes ($GFAP^{+}$ cells, red) and microglia ($IBA\text{-}1^{+}$ cells, red) within the brain.

The levels of vacuolation, deposition of PrP^{Sc} and accumulation of activated astrocytes and microglia were slightly lower in the brains of transgenic animals in comparison to WT controls. However all characteristic hallmarks of TSE neuropathology are present.

These data confirm that transgenic animals are equally susceptible to TSE disease as WT counterparts. Therefore, any differences in TSE pathogenesis observed within the peripheral lymphoid tissues are due to specific removal of PrP^{C} expression on the FDCs and not a general resistance to TSE disease. Scale bars 500 μm. Sections counterstained with haematoxylin.

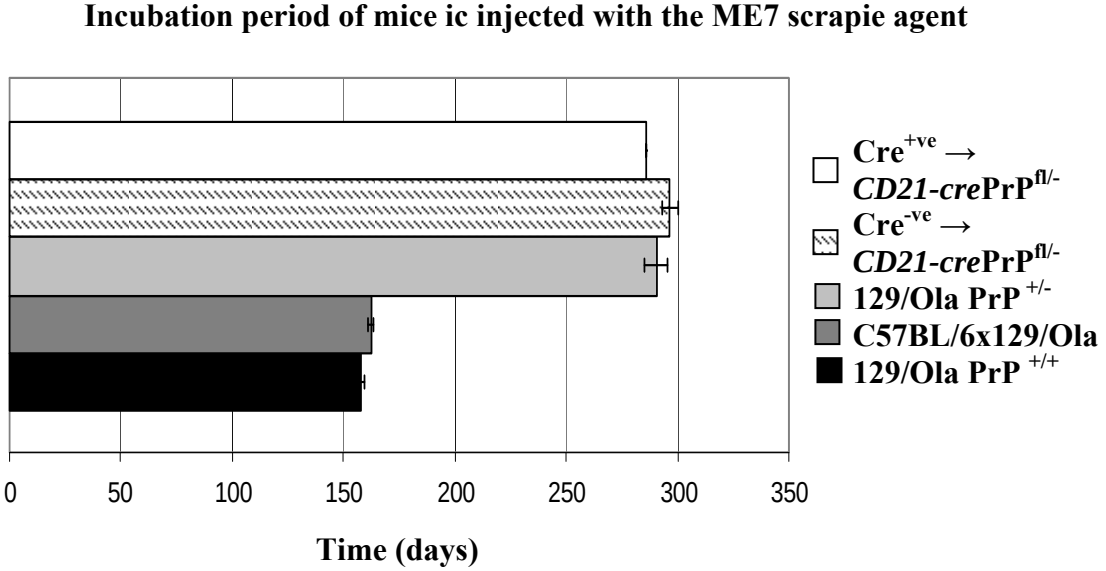


Figure 5.9 Mean duration of incubation periods of clinically-scrapie-affected mice inoculated ic with the ME7 scrapie agent

Incubation period of disease in transgenic and WT control lines after ic inoculation with the ME7 scrapie agent. Each bar represents the mean +/- the standard error of the mean for groups of 3-5 mice.

129/Ola and C57BL/6x129/Ola WT mouse lines which are *Prnp*$^{+/+}$ have incubation periods of approximately 160 d after ic inoculation with the ME7 scrapie agent. Transgenic *CD21-cre*PrP$^{fl/-}$ lines have an incubation period of around 280 d. This is in line with the disease incubation observed in129/Ola Prnp$^{+/-}$ mice which also express half copy number levels of PrP^{C}.

mice were also inoculated ic as controls. Following infection, both WT and transgenic animals developed clinical TSE disease with positive spongiform pathology in the brain. The classical hallmarks of TSE infection in the brain are deposition of disease-associated PrP^{Sc}, gliosis and spongiform pathology with vacuolation. Immunolabelling confirmed PrP^{d}, microglia (IBA-1^{+} cells) and activated astrocytes ($GFAP^{+}$ cells) were all present in the brains of WT and transgenic animals. These data confirm that *CD21-cre*$PrP^{fl/-}$ transgenic mice were fully susceptible to TSE disease when injected directly into the brain (Fig 5.8). However the neuropathology in the brains of transgenic animals appeared to be at lower than that found in WT controls, possibly due to the transgenic mice only expressing half copy number levels of *Prnp* or due to mouse strain differences. Transgenic *CD21-cre*$PrP^{fl/-}$ animals all succumbed to the clinical signs of scrapie around 280 days post-ic inoculation with the ME7 scrapie agent (Fig 5.9). This is in line with previous transmissions of the ME7 scrapie agent into 129/Ola $Prnp^{+/-}$ mice undertaken at this instititute, which also express half copy number expression levels of *Prnp* (Manson, Clarke et al. 1994b).

These data demonstrate that the transgenic lines used are equally susceptible as WT mice to the ME7 scrapie agent when infection was established directly in the brain by ic injection. Furthermore, insertion and expression of the *Cre* and $Prnp^{fl}$ transgenes also appeared to have had no detectable effect on the neuropathology in the CNS.

5.3.5 No PrP^{Sc} accumulation upon FDCs in the spleens of clinically-scrapie-affected mice with FDC-restricted PrP^{C} ablation

Following exposure to the ME7 scrapie agent, high levels of PrP^{Sc} are maintained on FDCs for the duration of the incubation period; even after ic exposure (Brown, Stewart et al. 1999). The spleens from the clinically-scrapie-affected, ic injected mice were also assessed to determine the cellular sites of PrP^{Sc} accumulation. As anticipated, spleens from PrP^{C}-expressing WT animals showed heavy accumulation of PrP^{d} in the follicles as determined by immunolabelling (Fig 5.11). This deposition was confirmed to be PrP^{Sc} by the PET blot method (Fig 5.12). However, in spleens with Cre-expressing, PrP^{C} deficient FDCs, little to no PrP^{d} could be detected. Where PrP^{d} was detected it appeared to be mostly associated with TBMs rather than the FDC networks (Fig 5.11). Furthermore, this pattern of immunolabelling could not be confirmed as PrP^{Sc} by PET blots (Fig 5.12). This suggests that although animals with Cre-expressing, PrP^{C} deficient FDCs are susceptible to ME7 infection in the CNS, animals with terminal clinical disease are still unable to replicate the TSE agent in the periphery when FDCs do not express PrP^{C}. This confirms that even at clinical disease without PrP^{C}-expressing FDCs, the scrapie agent is unable to accumulate upon FDCs in the lymphoid tissue and is instead cleared by neighbouring TBMs.

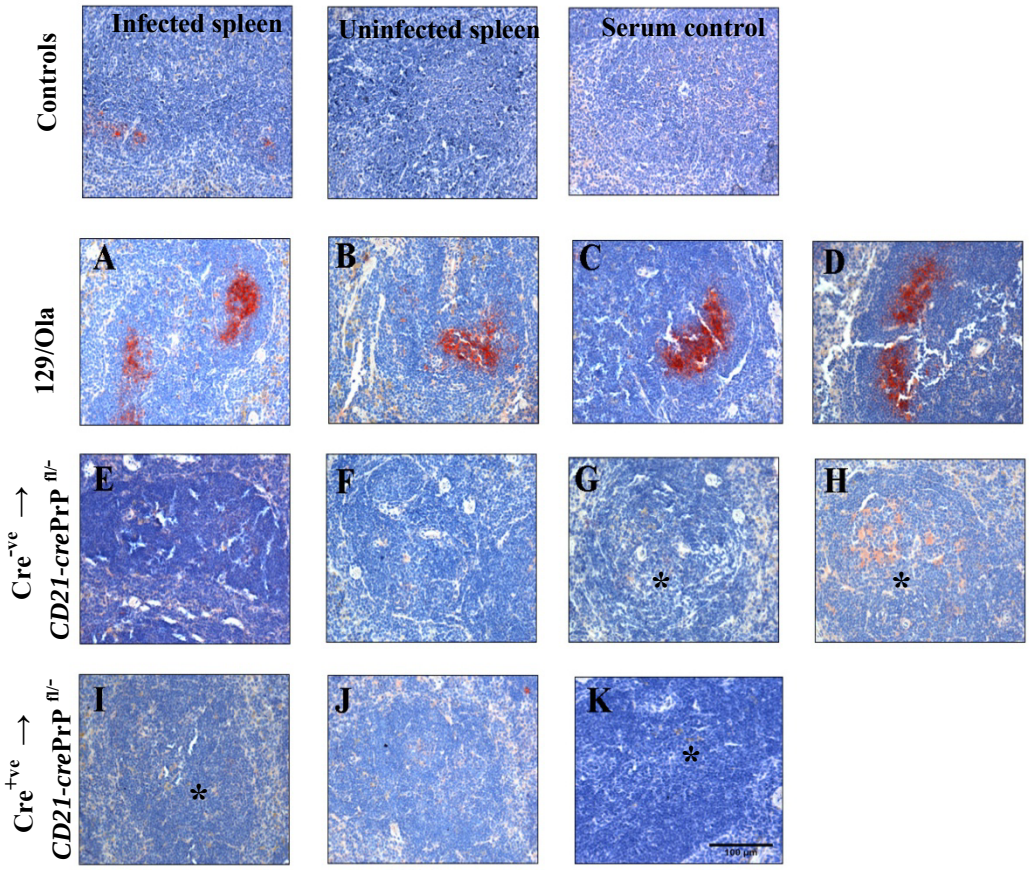


Figure 5.11 PrP^d immunolabelling in the spleens of clinically-scrapie-affected mice ic inoculated with the ME7 scrapie agent

Animals were inoculated ic with ME7 scrapie and left to develop terminal disease. Spleens were immunolabelled to detect PrP^d (PAb 1B3, red).

Spleens from WT animals with Cre-deficient, PrP^C-expressing FDCs (A-D) show intense PrP^d immunolabelling on the FDC networks (▲) within the B lymphocyte follicles. In contrast, spleens from transgenic animals with Cre-expressing, PrP^C deficient FDCs (E-K) show no PrP^d labelling on the FDC networks. Instead, the only PrP^d detected is found within TBMs (*) in the follicle. This suggests the scrapie agent is unable to replicate on PrP^C deficient FDCs even when animal has clinical TSE disease. Scale bar 100 µm. Sections counterstained with haematoxylin,blue.

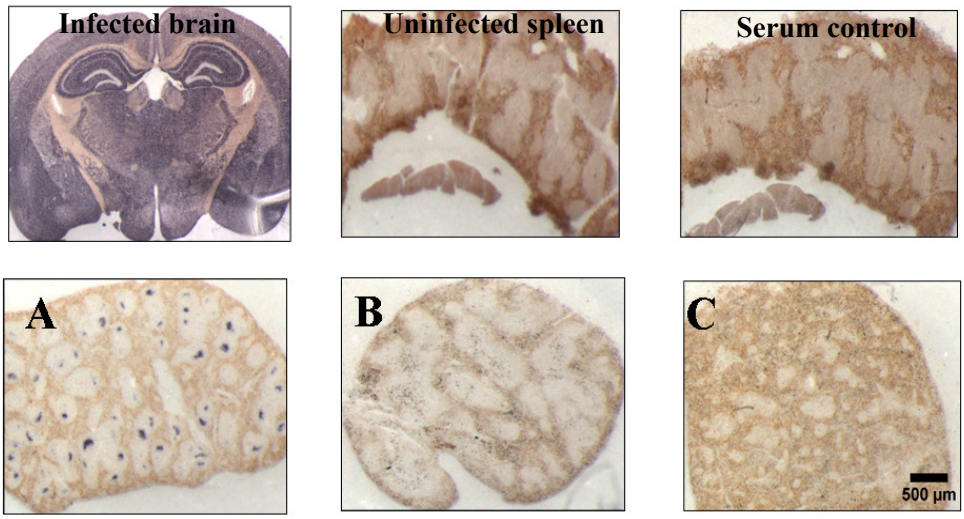


Figure 5.12 PET blots analysis of PrP^{Sc} accumulation in spleens from clinically-scrapie-affected mice ic inoculated with the ME7 scrapie agent
Paraffin-embedded sections on nitrocellulose membrane were treated with PK to remove any native PrP^{C}, then immunolabelled (PAb, 1B3, blue/black) to detect any remaining PrP^{Sc}. Labelling in ic injected transgenic animals **B,** Cre^{-ve}→ *CD21-cre*$PrP^{fl/-}$; **C,** Cre^{+ve}→ *CD21-cre*$PrP^{fl/-}$ was compared with that of **A**, 129/Ola WT animals. Scale bar 500 µm

In WT spleens with Cre-deficient, PrP^{C}-expressing FDCs (A), intense PrP^{Sc} labelling (➔) can be confirmed in the B lymphocyte. However, in transgenic spleens with Cre-expressing, PrP^{C} deficient FDCs (B and C), immunohistochemistry had shown PrP^{d} was present only within the TBMs (Fig 5.10). This pattern of immunolabelling is not readily detected after PK treatment and therefore could not be confirmed to be the disease-associated PrP^{Sc}.

5.4 Discussion

The *CD21-cre*PrP$^{fl/-}$ mouse line, with irradiation and reconstitution with non-Cre-expressing bone marrow, was previously shown to allow the removal of PrP^{C} specifically on FDCs with no other adverse effects on the lymphoid tissues (Chapter 4). In this chapter the *CD21-cre*PrP$^{fl/-}$ mouse line was injected ip with the ME7 scrapie agent to determine if PrP^{C}-expressing FDCs simply acquire the scrapie agent after replication on another cell type within the lymphoid tissue. As host expression of PrP^{C} is essential for TSE agent pathogenesis (Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997), if FDCs themselves actively replicate the TSE agent, then PrP^{Sc} accumulation should not be possible when PrP^{C} expression is removed exclusively on the FDC network. In contrast, if FDCs only acquire TSE agents, high levels of PrP^{Sc} will accumulate upon the FDC, even in the absence of PrP^{C} expression.

Scrapie agent accumulation in lymphoid tissues was determined by immunolabelling for PrP^{d} and PrP^{Sc}. Previous studies have shown that PrP^{Sc} co-purifies with infectivity in tissues from scrapie-affected animals and has been confirmed in many studies to be a reliable marker of TSE disease in the lymphoid tissue (Bolton, McKinley et al. 1982; McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009). Abnormal, pathological accumulations of PrP detected by immunohistochemistry were termed PrP^{d}, as the antibodies used cannot distinguish between PrP^{C} and PrP^{Sc}. Any PrP^{d} present was subsequently verified to be PrP^{Sc} if immunolabelling was still detected after PK treatment using the PET blot method (Section 2.4.3). Previous studies have

shown that PrP^{Sc} and infectivity accumulates in the lymphoid tissues long before detection in the CNS and deposition on the FDC network can be seen easily at 4 weeks post peripheral inoculation (Brown, Stewart et al. 1999). For this reason, animals were culled at 35 and 70 dpi to assess the early pathogenesis of the ME7 scrapie agent in transgenic and control lines. However, as animals in this study are expressing only half copy number levels of *Prnp*, less deposition of PrP^{Sc} would be expected on the FDC networks than seen in equivalent studies using $Prnp^{+/+}$ animals.

Animals with Cre-deficient, PrP^{C}-expressing FDCs had detectable PrP^{d} in the spleen at 35 dpi and this accumulation intensified by 70 dpi suggesting active replication of the scrapie agent upon the PrP^{C}-expressing FDCs. This is in agreement with previous studies which suggested that PrP^{C}-expressing FDCs are essential for replicating the scrapie agent (McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, McGovern et al. 2002; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009). However in animals with Cre-expressing, PrP^{C} deficient FDCs, deposition was only seen on occasional FDC networks at 35 dpi. By 70 dpi PrP^{d} could no longer be found on the FDC networks and is instead located within TBMs in the follicle. These data suggest that the scrapie agent initially locates to the FDCs network independently of PrP^{C} expression on the FDCs. Previous data indicates that the scrapie agent is opsonised by complement components and is trapped on the FDC network as an immune complex bound by complement and Fc receptors (Klein, Kaeser et al. 2001; Mabbott, Bruce et al. 2001; Zabel, Heikenwalder et al. 2007). With no PrP^{C} present on the FDCs, data in this chapter shows that the scrapie agent is unable to replicate and is cleared from the FDC network by TBMs in the surrounding

follicle. Previous studies have shown that TBMs are capable of taking up immune-complex coated portions of FDC membrane, also known as iccosomes, therefore this is a possible mechanism by which TBMs remove the scrapie agent from the FDC surface (Szakal and Tew 1992; Sandberg, Al-Doujaily et al. 2011). As Cre-expressing, PrP^{C} deficient FDCs have no deposition of PrP^{Sc} on their surface, it can be concluded that FDCs do not accumulate the TSE agent from the other cells in the lymphoid tissue, such as neurones, stromal cells and lymphocytes which retain PrP^{C} expression in this model.

CHAPTER 6

Characterisation of the *CD21-cre*PrP$^{stop/-}$ mouse line

6.1 Abstract

The *CD21-cre* mouse line has been used to switch off PrP^C expression specifically on FDCs and subsequently used to show that PrP^C expressing FDCs are required for replication of the scrapie agent in the lymphoid tissues (Chapter 4 and 5). To complement this model, the *CD21-cre* mouse line was subsequently crossed with a line which contains a floxed β-geo stop cassette inserted before the coding region of PrP^C. This *CD21-cre*$PrP^{stop/-}$ line allows PrP^C expression to be switched on under control of the *Cr2* promoter. In the absence of Cre expression, the floxed stop cassette prevents transcription of the open reading frame of the *Prnp* gene. However in CD21-expressing cells, Cre-mediated recombination removes the stop cassette and PrP^C expression is switched on. Irradiation and reconstitution of *CD21-cre*$PrP^{stop/-}$ mice with non-Cre-expressing bone marrow allows PrP^C expression to be switched on exclusively on CD21-expressing FDCs. *CD21-cre*$PrP^{stop/-}$ mice were fully characterised to determine within which cells PrP^C was switched on and to ensure that the transgenes had no additional effects on lymphoid tissue microarchitecture that could have an impact on scrapie pathogenesis. Characterisation of the *CD21-cre*$PrP^{stop/-}$ mouse demonstrated that PrP^C was efficiently and exclusively expressed on FDCs under control of the *Cr2* promoter. Furthermore, insertion and expression of the transgenes had no adverse effects on the microarchitecture of the lymphoid tissue. These data suggest that the *CD21-cre*$PrP^{stop/-}$ mouse will be a useful model to determine if PrP^C expression exclusively on FDCs is sufficient to support replication of the scrapie agent in the lymphoid tissue.

6.2 Introduction

Previous work in this thesis has shown that if PrP^C expression is removed specifically on the FDCs, the scrapie agent is unable to replicate in the spleen and accumulate on FDC surface (Chapter 5). In this chapter, a transgenic mouse line was created to allow PrP^C expression to be switched on exclusively on the FDC network, which would subsequently be used in Chapter 7 to determine if PrP^C expression on FDCs alone was sufficient to allow scrapie agent replication in the spleen.

The coding region for PrP^C is contained within exon 3 of the *Prnp* gene (Chapter 1, Fig 1.1). In $PrP^{stop/-}$ mice, the coding region of *Prnp* is preceded by a β-geo stop cassette flanked by *loxP* sites (Tuzi, Clarke et al. 2004). The $PrP^{stop/-}$ mouse line was crossed with the *CD21-cre* mouse to allow PrP^C to be switched on under control of the *Cr2* promoter (Fig 2.3). Based on data from the *CD21-creROSA26* and *CD21-cre*$PrP^{fl/-}$ lines (Chapter 3 and 4, respectively) the *CD21-cre*$PrP^{stop/-}$ line, together with lethal γ-irradiation and reconstitution with non-Cre-expressing bone marrow, should restrict PrP^C expression exclusively to FDCs. In this chapter, the *CD21-cre*$PrP^{stop/-}$ line was characterised to determine if PrP^C was successfully switched on specifically on the FDC networks and also that insertion of the transgenes had no additional effects on the lymphoid tissue. Analysis of the *CD21-cre*$PrP^{stop/-}$ line confirmed that PrP^C was efficiently expressed on Cre-expressing FDCs. Furthermore, no PrP^C expression was detected on non-CD21-expressing cells in the lymphoid tissues such as the nerves. In addition, there were no differences in the michroarchitecture of the lymphoid component of the spleen which could have potentially impacted on scrapie pathogenesis. From these data, it can be concluded that the *CD21-cre* PrP^{fl} line will

be a useful tool to study scrapie pathogenesis when PrP^C expression has been switched off exclusively on FDCs.

6.3 Results

6.3.1 Production of *CD21-cre*PrP$^{stop/-}$ mouse line

The *CD21-cre*PrP$^{-/-}$ mouse line, which was created during previous work to make the *CD21-cre*PrP$^{floxed/-}$ mouse line (Section 2.1.3 and 4.3.2), was crossed with PrP$^{stop/-}$ line to create the *CD21-cre* PrP$^{stop/-}$ mice. The PrP$^{stop/-}$ line contains a floxed stop cassette inserted into the *Prnp* gene prior to exon 3 which contains the ORF (Tuzi, Clarke et al. 2004). In the absence of Cre, cells in this animal cannot express PrP^{C} due to the presence of the stop cassette before the protein coding sequence in exon 3. However in cells which express CD21, Cre recombinase is expressed, the stop cassette is removed and PrP^{C} expression is switched on. The *Prnp* ^{stop} allele is lethal at homozygosity (Tuzi, Clarke et al. 2004), therefore, no attempts were made to create a homozygous *CD21-cre*PrP$^{stop/stop}$ mouse line.

To restrict PrP^{C} expression exclusively to FDCs, *CD21-cre*PrP$^{stop/-}$ animals were lethally γ-irradiated and given bone marrow from age- and sex-matched Cre negative littermates (termed Cre^{-ve} →*CD21-cre*PrP$^{stop/-}$). Experimental control groups were also produced as in the *CD21-cre*PrP$^{floxed/-}$ experimental lines. Firstly, *CD21-cre*PrP$^{stop/-}$ mice given bone marrow from Cre^{+ve} littermates, (termed Cre^{+ve} →*CD21-cre*PrP$^{stop/-}$ mice), in which PrP^{C} will be switched off in both FDCs and CD21^{+} B lymphocytes. Also, PrP$^{fl/-}$ mice were given bone marrow from *CD21-cre* PrP$^{stop/-}$ mice, (termed Cre^{+ve} → PrP$^{stop/-)}$, in which PrP^{C} expression is switched on in CD21^{+} B lymphocytes only. The final control group was PrP$^{WT/-}$ mice given PrP$^{WT/-}$ bone marrow. The copy number of the *Prnp* gene has been shown to have a strong

influence in scrapie incubation period, with $PrP^{WT/-}$ heterozygous mice having almost double the incubation time of $PrP^{WT/WT}$ homozygotes (Tew and Mandel 1979). Therefore, this control group was included due to the *CD21-cre*$PrP^{fl/-}$ experimental line having half copy number levels of PrP^{C}.

Bone marrow chimera status of experimental animal groups was confirmed by PCR analysis (Fig 6.1). DNA was extracted from blood, tail and spleen and was genotyped for both expression of *Cre* and *Prnp*^{stop} with and without Cre-mediated recombination of the *Prnp*^{stop} DNA. This analysis confirmed that the host/ donor genotypes were as expected and also that recombination of the *Prnp*^{stop} DNA only occurred in tissues which contained both Cre expression and CD21-expressing cells.

6.3.2 Insertion and expression of transgenes and bone marrow reconstitution have no effect on lymphoid tissue michroarcitecture

In addition to confirming that Cre-mediated recombination was restricted to CD21-expressing cells and was occurring efficiently, it was also essential to ensure that there were no other changes to the lymphoid tissue that could mediate an effect on scrapie pathogenesis. Spleens from all four groups of experimental mice were immunolabelled to detect B lymphocyte subsets, T lymphocytes, FDCs and classical dendritic cells and compared with immunolabelling of spleens from WT animals (Fig 6.2)

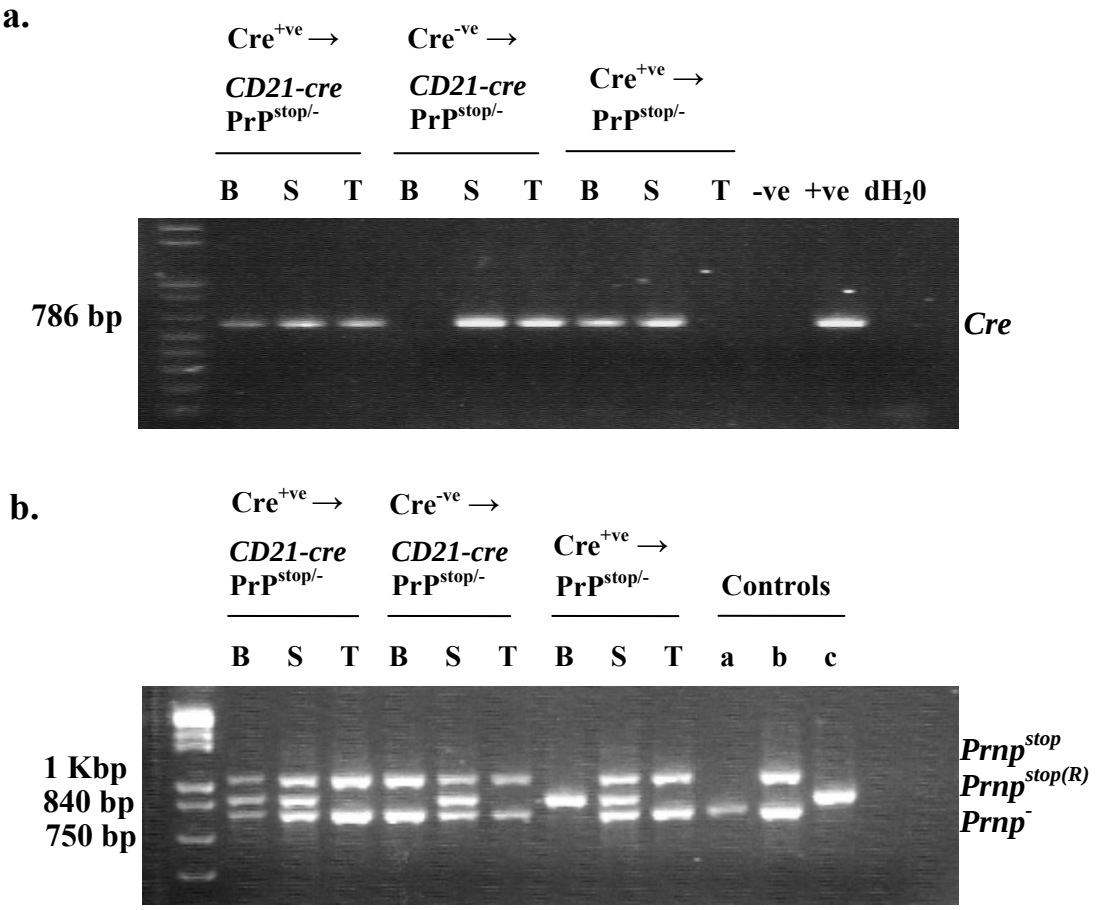


Figure 6.1 Genotypes of *CD21-cre*PrP$^{stop/-}$ animals used for characterisation and subsequent scrapie experiments
DNA was extracted from blood (B), spleen (S), and tail (T) from 6 animals from each of the transgenic lines to be used in subsequent scrapie experiments. One example of genotype results for Cre^{+ve} →*CD21-cre*PrP$^{stop/-}$, Cre^{-ve} →*CD21-cre*PrP$^{stop/-}$ and Cre^{+ve} →PrP$^{stop/-}$ lines is shown on the above gel.

a. The *Cre* transgene was present in the spleen and tail of *CD21-cre*PrP$^{stop/-}$ mice but was only present in the blood if Cre^{+ve} donor bone marrow had been given. PrP$^{stop/-}$ mice had no *Cre* transgene detected in tail DNA, however *Cre* was present in the spleen and blood due to Cre^{+ve} bone marrow given.

b. Tissues from all animals are heterozygous for *Prnp*$^{stop/-}$ however, recombination of the *Prnp*^{stop} DNA (*Prnp*$^{stop(R)}$) was only present in tissues that contain both the *Cre* transgene and CD21-expressing cells. Control DNA was from *Prnp*^{WT} (a), *Prnp*$^{stop/-}$ (b) and *Prnp*$^{stop(R)}$ DNA extracted from tails.

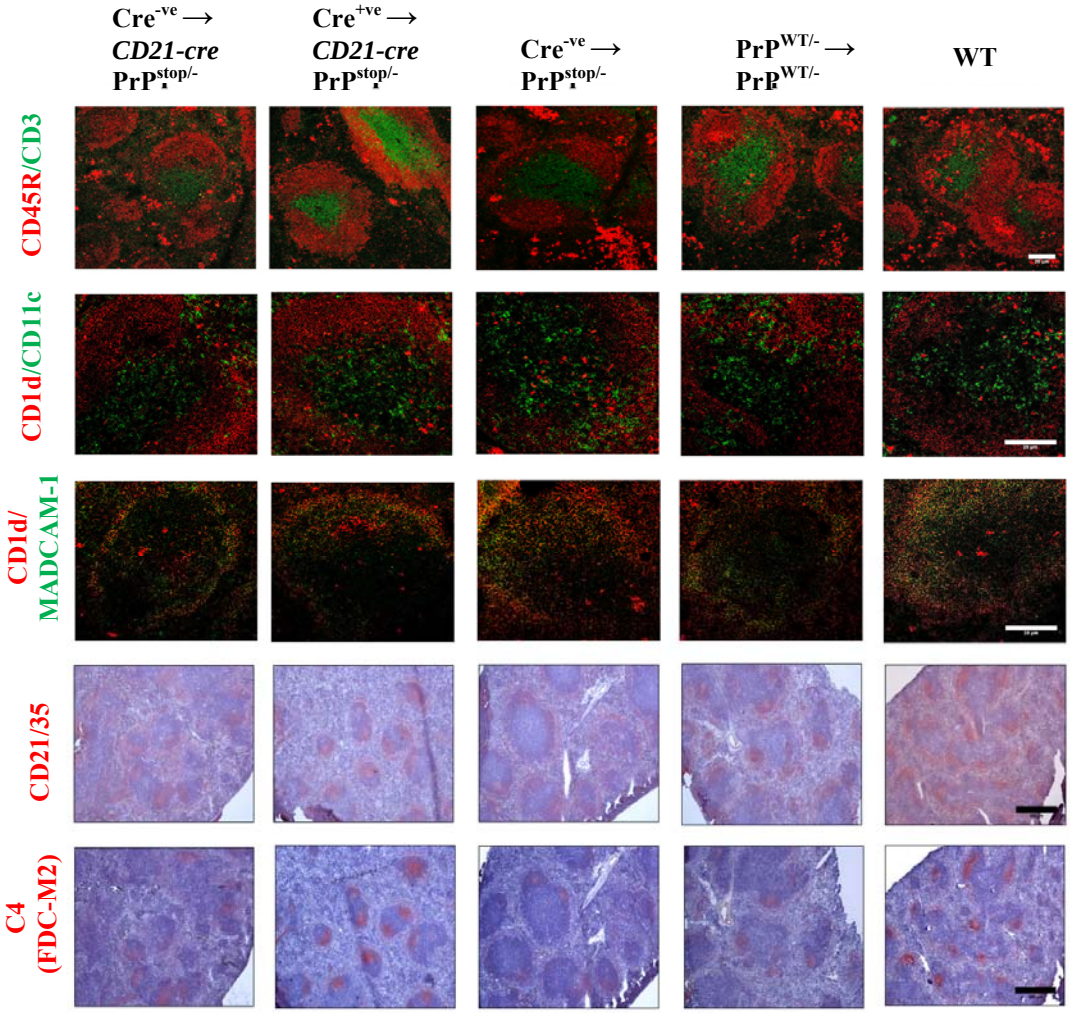


Figure 6.2 Insertion and expression of the transgenes and irradiation and bone marrow reconstitution has no effect on the microarchitecture of peripheral lymphoid tissues as determined by immunolabelling

Frozen spleen sections from all experimental transgenic mouse lines were immunolabelled for B lymphocyte subsets (CD45R andCD1d), T lymphocytes (CD3), FDC (C4, red and CD21/35, red), DCs (CD11c) and marginal zone cells (MADCAM-1). Comparison of sections from transgenic animals with WT controls showed no differences in the number or location of cell subsets within the spleen. Scale bars on fluorescent images 100 μm. Scale bars on light microscopy images 500 μm and sections counterstained with haematoxylin, blue.

No differences could be seen in intensity or location of immunolabelling for each cell type between WT and experimental spleens. It can be concluded that neither expression of the transgenes nor irradiation and bone marrow reconstitution has any significant effect on the michroarchitecture of the lymphoid tissue.

6.3.3 PrP^C immunolabelling is present on Cre-expressing FDCs

Spleens from all experimental and control lines were immunolabelled for PrP^C expression to determine within which cells PrP^C protein expression was switched on. Within the lymphoid tissues, PrP^C can be detected on FDCs and peripheral nerves using immunolabelling. Therefore, to determine if PrP^C expression was switched on specifically on the FDC network, spleen sections were fluorescently immunolabelled for PrP^C, FDCs via CD35 expression and tyrosine hydroxylase (TH) expressing peripheral nerves. Co-localisation of fluorescent labelling was then quantified using ImageJ image analysis software. Animals which expressed Cre showed PrP^C immunolabelling present on the FDC networks but this was absent on the Cre-deficient peripheral nerves (Fig 6.3). Quantification of co-localised pixels showed that the co-localisation detected was true co-localisation and not background due to random association of pixels ($p=1.2 \times 10^{-26}$*; 9.1×10^{-25}** and 1.3×10^{-23}***, Fig 6.4). Animals with PrP^C expression switched on specifically on FDCs had statistically significantly higher levels of PrP^C staining in comparison to non-Cre expressing spleens ($p<1.1 \times 10^{-25}$, Fig 6.4).

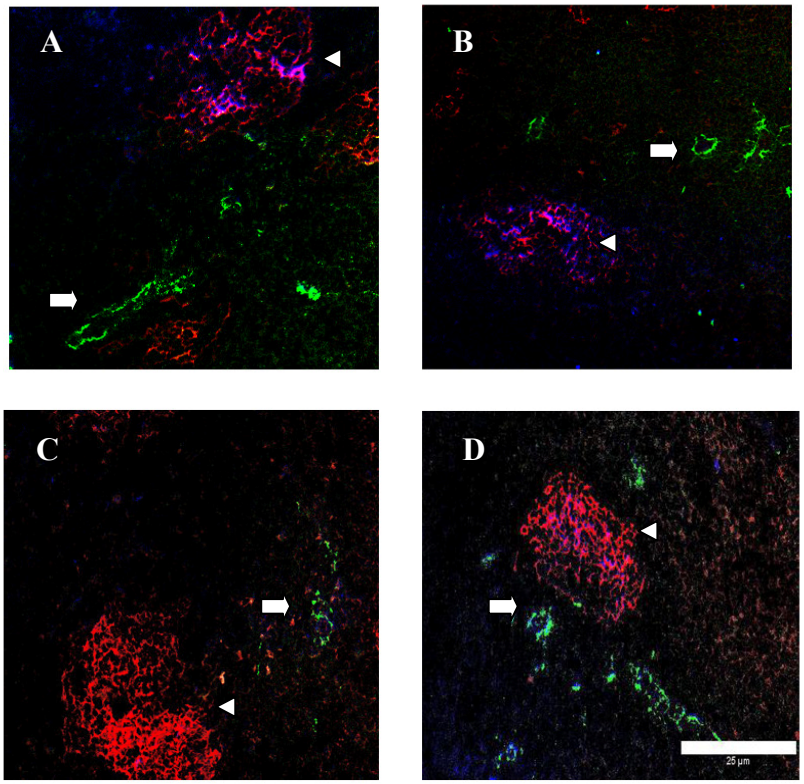


Fig 6.3 PrP^C is switched on specifically on FDC networks of animals which express Cre

Immunolabelling of PrP^C (blue) on FDC networks ◁ (CD35/red) and peripheral nerves ⇨ (TH/green) in spleen sections from transgenic mouse lines. Animals were lethally γ-irradiated, received donor bone marrow 24 h later and were culled 100 days post reconstitution to allow replacement of all host-derived lymphocytes. Spleen sections from 6 animals from each line were immunolabelled and analysed. *CD21-cre*$PrP^{stop/-}$ animals which received Cre^{-ve} (A) or Cre^{+ve} (B) bone marrow had detectable PrP^C labelling on the FDC networks however PrP^C labelling was not present on the peripheral nerves. This demonstrates that expression of PrP^C has been switched on specifically on the Cre-expressing FDCs.

$PrP^{stop/-}$ animals which received Cre^{+ve} bone marrow (C) showed no PrP^C immunolabelling on FDCs or nerves. B lymphocyte expression of PrP^C is not detectable by immunolabelling. $PrP^{WT/-}$ animals which received $PrP^{WT/-}$ bone marrow (D) show PrP^C labelling on both FDC networks and peripheral nerves. These data demonstrate that PrP^C has been switched on specifically on the FDCs of animals which express Cre under the *Cr2* promoter. Scale bar 100 µm.

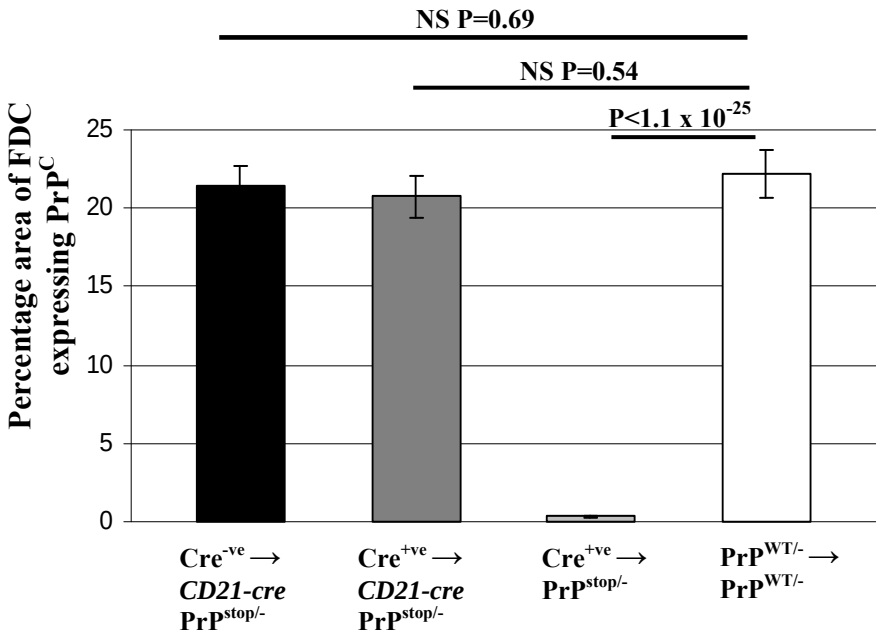


Fig 6.4 Expression of Cre in FDCs under the CD21 promoter removes PrP^C immunolabelling on FDC networks

Frozen spleen sections were immunolabelled for PrP^C, FDC and peripheral nerves. For each mouse line, spleens were taken from 6 animals. Two sections, 50 μm apart, were immunolabelled for each spleen and 4 images per section were taken. This resulted in the analysis of 48 images for each mouse line using ImageJ image analysis software. For each image the number of pixels of each colour were counted using the multiple colour analysis macro allowing values to be obtained for total number of red (FDC) blue (PrP^C) and magenta (PrP^C co-localised with FDC) pixels per image. These values allowed the calculation of the average percentage of FDC area co-localised with PrP^C. In *CD21-cre*$PrP^{stop/-}$ animals and $PrP^{WT/-}$ animals, which have Cre-expressing FDCs this value is significantly higher than $PrP^{stop/-}$ animals which do not have Cre-expressing FDCs. These data show that PrP^C has been switched on specifically on the Cre-expressing FDCs as anticipated.

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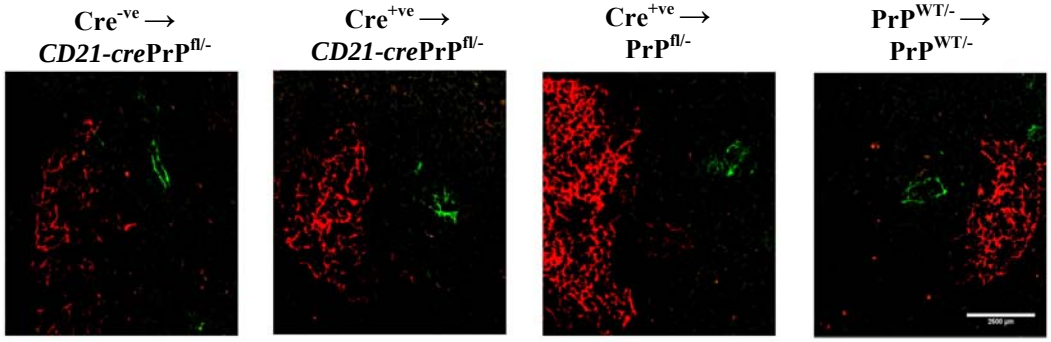
6.3.4 Insertion and expression of transgenes and irradiation and bone marrow reconstitution have no effect on the distance between FDC networks and peripheral nerves in the spleen

Previous studies have shown that the distance between peripheral nerves and FDC networks can influence scrapie incubation period after peripheral inoculation (Glatzel, Heppner et al. 2001; Prinz, Heikenwalder et al. 2003). Therefore immunolabelling was used to further characterise the microarchitecture of lymphoid tissues of transgenic mice by measuring the distance between peripheral nerves and FDCs in the spleen. Frozen sections were immunolabelled for FDCs and peripheral nerves and LSM image browser software was used to measure the distance between them (Fig 6.5). Comparison of the average distance between FDCs and the nearest peripheral nerve showed there was no statistically significant difference in the distance between FDCs and peripheral nerves between transgenic spleens in comparison to WT counterparts ($p=0.932$, Fig 6.5).

a.



b.

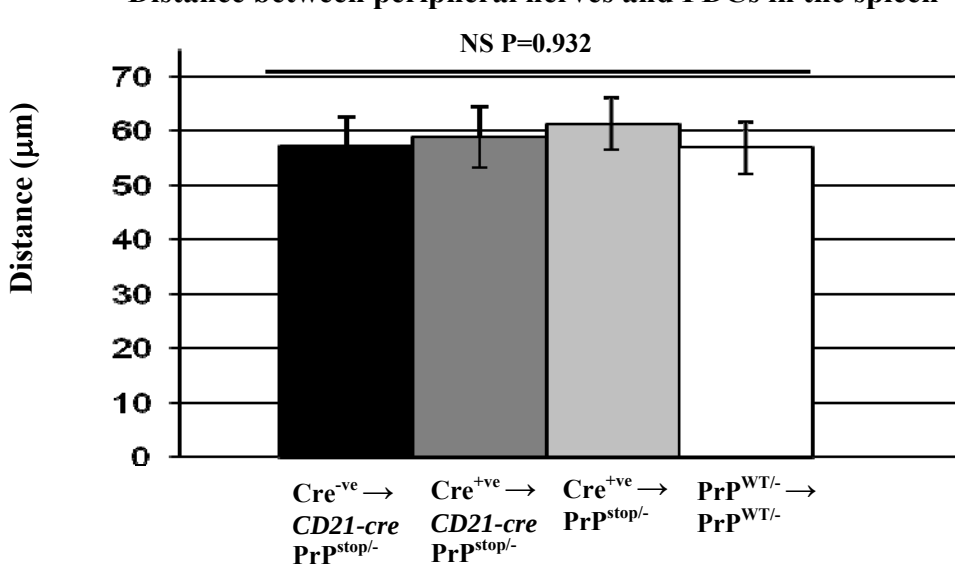


Fig 6.5 Insertion and expression of the transgenes has no effect on the distance between peripheral nerves and FDCs in the spleen

a. Frozen spleen sections from transgenic and WT lines were immunolabelled for FDC networks (CD35/red) and peripheral nerves (TH/green). For each mouse line, spleens were taken from 6 animals. Two sections, 50 μm apart, were immunolabelled for each spleen and 4 images per section were taken. 48 images were analysed for each mouse line. Scale bar 50 μm.

b. The distance between the FDC networks and peripheral nerves was measured using LSM image browser software (Zeiss). The average distance between nerves and FDCs was calculated for each mouse line. Analysis using a one-way anova test determined there was no significant difference in the distance between the FDC networks and peripheral nerves in the spleens of transgenic lines in comparison to WT controls (p=0.932).

6.4 Discussion

The *CD21-cre* line was crossed with a $PrP^{stop/-}$ line to create a model where PrP^{C} expression could be switched on exclusively on FDCs. In this chapter the *CD21-cre*$PrP^{stop/-}$ line was characterised to establish if this mouse model would be an efficient tool to determine whether PrP^{C} expression exclusively on FDCs is sufficient to allow replication of the scrapie agent within the lymphoid tissue. The characterisation studies carried out on this line indicate that PrP^{C} was switched on specifically on Cre-expressing FDCs and not on other non-Cre-expressing cells within the lymphoid tissue such as the nerves. Furthermore, insertion and expression of the transgenes had no additional effects on the lymphoid tissue that could influence scrapie pathogenesis.

Homozygous expression of the $Prnp^{stop}$ allele is embryonically lethal. Therefore *CD21-cre*$PrP^{-/-}$ mice were crossed once with the $PrP^{stop/-}$ mice and selected *CD21-cre*$PrP^{stop/-}$ progeny were used in subsequent characterisation. *Prnp* expression levels have previously been shown to have a crucial influence on scrapie incubation period (Manson, Clarke et al. 1994a). Therefore, in subsequent scrapie infection experiments (Chapter 7), $PrP^{WT/-}$ mice will be used as a control line for the half copy number expression levels of PrP^{C} present on the FDCs of the *CD21-cre*$PrP^{stop/-}$ mice.

Characterisation of the *CD21-cre*$PrP^{stop/-}$ mice showed that PrP^{C} expression was efficiently and exclusively expressed on FDCs. PCR analysis of DNA extracted from spleens confirmed the presence of the *Cre* transgene and recombination of the $Prnp^{stop}$ transgene. Furthermore, immunolabelling of spleen sections confirmed that PrP^{C} was

expressed on FDCs but not present on non-CD21 expressing cells such as peripheral nerves. Previous studies using the CD21-cre line, which measured 96% of floxed DNA was recombined in FDCs of CD21-cre mouse line crosses (Victoratos, Lagnel et al. 2006). This type of analysis was not carried out on *CD21-cre*PrP$^{stop/-}$ mice, however PCR and immunohistochemical analysis showed efficient recombination of the floxed DNA.

Depletion of various lymphoid cell subsets other than FDCs has been shown to delay the onset of peripherally acquired scrapie (Klein, Frigg et al. 1997; Raymond, Aucouturier et al. 2007). Therefore, characterisation of the lymphoid tissues of transgenic mice was essential to ensure that the only factor affecting scrapie pathogenesis in *CD21-cre*PrP$^{stop/-}$ mice was the expression of PrP^{C}. Various cell subsets were immunolabelled on spleen sections from transgenic experimental and WT lines to determine if insertion of the transgenes or irradiation had any effect on lymphoid tissue michroarchitecture. No observable difference could be seen in the number or location of B lymphocytes, T lymphocytes or classical dendritic cells or in the integrity of the marginal zone in the spleen.

Changes in the number and maturation status of the FDC networks in the lymphoid tissue has been shown to have a critical influence on peripheral scrapie pathogenesis (Brown, Stewart et al. 1999; Mabbott, Williams et al. 2000a; Mabbott, McGovern et al. 2002; Mabbott, Young et al. 2003; Prinz, Heikenwalder et al. 2003). Analysis of the *CD21-cre*PrP$^{fl/-}$ line (Chapter 4) showed there were no significant differences in the number, area and function of FDC networks of transgenic mice in comparison to WT controls. It was not considered to be essential to repeat these extensive studies on

FDCs of *CD21-cre*PrP$^{stop/-}$ mice, however immunolabelling of spleen sections from this line showed no observable differences in the presence of various FDC markers in comparison to WT controls. The positioning of FDC networks within the spleen with regard to their proximity to peripheral nerves can also influence the incubation period of peripherally acquired scrapie (Glatzel, Heppner et al. 2001; Prinz, Heikenwalder et al. 2003). This was measured in spleen sections from transgenic experimental lines and WT controls and no significant difference was measured in the distance between the FDC networks and nerves.

These data show that neither the insertion and expression of the *Cre* or *Prnp*^{stop} transgenes nor irradiation and bone marrow reconstitution in experimental mice, has any significant effect on the microarchitecture of the spleen or the number or distribution of various cell subsets found within. Previous studies using the *CD21-cre* mice also did not report any toxic effects caused by the *Cre* transgene and showed no differences in FDC and B lymphocyte numbers or location in the spleen (Kraus, Alimzhanov et al. 2004; Victoratos, Lagnel et al. 2006; Schenten, Kracker et al. 2009). Additionally, characterisation of the PrP$^{-/-}$ line has shown no overt phenotype even though PrP^{C} is ubiquitously expressed (Manson, Clarke et al. 1994a).

FDCs have the ability to acquire proteins on their surface which they themselves do not express, for example Class II MHC (Gray, Kosco et al. 1991; Denzer, van Eijk et al. 2000). Although PrP^{C} has been shown to be present on the FDC surface via immunolabelling and electron microscopy, it is possible that FDCs acquire the expression of PrP^{C} from other cell types within the lymphoid tissues. Switching on *Prnp* expression exclusively in FDCs has determined that FDCs themselves do

actively express relatively high levels of PrP^C and are not acquiring this expression from other cells. This is in agreement with previous studies using bone marrow chimeric mice. Mice were lethally γ-irradiated and reconstituted with $PrP^{-/-}$ bone marrow, however host derived FDCs remained $PrP^{+/+}$ suggesting that FDCs themselves expressed PrP^C (Brown, Stewart et al. 1999).

Characterisation of the CD21-cre$PrP^{stop/-}$ mouse line has shown that PrP^C is efficiently switched on in Cre-expressing FDCs but is not present on other cell types within the lymphoid tissues. Furthermore, insertion and expression of the transgenes have no additional effects on the cellular composition or structure of the lymphoid tissue. Therefore the CD21-cre$PrP^{stop/-}$ mouse line is an effective model to determine if PrP^C expression exclusively by FDCs is sufficient to allow scrapie agent replication in the lymphoid tissues (Chapter 7). This will allow the role of the FDCs in scrapie pathogenesis to be fully investigated and to establish whether FDCs themselves actively replicate the scrapie agent.

CHAPTER 7

Pathogenesis of ME7 Scrapie in mice with PrP^C expression switched on exclusively on FDCs

7.1 Abstract

Data in this thesis (Chapter 5) has shown that PrP^{C} deficient FDCs are unable to replicate the scrapie agent. Without PrP^{C}–expressing FDCs, PrP^{Sc} appears to be removed from the FDCs by TBMs in the follicle and possibly degraded. In this Chapter experiments are designed to determine if PrP^{C} expression exclusively by FDCs in a *Prnp*$^{-/-}$ animal is sufficient to support replication of the scrapie agent in the lymphoid tissue.

The *CD21-cre*$PrP^{stop/-}$ transgenic mouse model was extensively characterised (Chapter 6) and it was confirmed that in this model, PrP^{C} expression was switched on exclusively and efficiently on FDCs. Furthermore, the insertion and expression of the transgenes had no adverse effects on the cellular composition or microarchitecture of the lymphoid tissues that could potentially impact on scrapie pathogenesis. *CD21-cre*$PrP^{stop/-}$ mice and control lines were inoculated ip with ME7 scrapie and culled at various time points after inoculation to assess disease progression. By 35 dpi all animals with PrP^{C} expression exclusively in FDCs, or in FDCs and $CD21^{+}$ B lymphocytes, showed low levels of PrP^{d} immunolabelling on the FDC network. At later time points, immunolabelling of PrP^{d} was more intense and could be confirmed as PrP^{Sc} using the PET blot method. These data show that PrP^{C} expression exclusively on FDCs is sufficient to support replication of the scrapie agent in the lymphoid tissues. Therefore it can be concluded that the FDCs themselves are actively replicating the scrapie agent and not acquiring it after replication on another cell type.

7.2 Introduction

Heavy accumulations of TSE-agent-specific disease-associated PrP^{Sc} occur upon the FDC networks in lymphoid tissues of scrapie-affected mice after peripheral exposure (McBride 1992; Brown, Stewart et al. 1999; Bruce 2000; Jeffrey, McGovern et al. 2000). FDCs are thought to have a role in replicating the scrapie agent within the lymphoid tissue, however as of yet, there has not been a suitable model which has been able to specifically assess the role of the FDCs in isolation from all other stromal, neural and lymphoid cells within the lymphoid tissue (Brown, Stewart et al. 1999; Montrasio 2000; Mabbott, Williams et al. 2000a; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Mohan, Bruce et al. 2005). Previous data in this thesis (Chapter 5) has shown that PrP^{C} ablation specifically on FDCs prevents the accumulation of the scrapie agent on the FDC surface in the lymphoid tissue. This suggests that FDCs are not simply accumulating the FDCs on their surface after replication on another cell type within the lymphoid tissue and instead actively replicate the scrapie agent themselves.

In this Chapter it was determined whether PrP^{C} expression exclusively on FDCs was sufficient to support scrapie agent replication within the lymphoid tissue. To address this issue, the *CD21-cre* mouse line was crossed with the $Prnp^{stop/-}$ mouse line to create a model in which PrP^{C} expression was switched on exclusively on CD21-expressing cells. These animals were subsequently irradiated and reconstituted with non-*Cre*-expressing bone marrow to restrict PrP^{C} expression exclusively to the FDC network. Characterisation of this compound transgenic model (Chapter 6) showed that PrP^{C} was efficiently and specifically expressed on the FDC networks of *CD21-*

*cre*PrP$^{stop/-}$ mice. Furthermore, no adverse effects on the status of FDC networks, or in the general michroarcitecture of the lymphoid tissue were observed. These data confirmed that the *CD21-cre*PrP$^{stop/-}$ model would be a useful tool to determine the role of PrP^{C}-expressing FDCs in scrapie pathogenesis and determine whether if PrP^{C} expression on exclusively on FDCs is sufficient to support scrapie agent replication in the peripheral lymphoid tissues. As host expression of PrP^{C} is essential for TSE agent pathogenesis (Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997), if FDCs themselves actively replicate the TSE agent, then PrP^{C} expression exclusively on the FDC network should be sufficient to enable TSE agent replication in the lymphoid tissues.

*CD21-cre*PrP$^{stop/-}$ mice were infected ip with the ME7 scrapie agent and culled at 35, 70 or 105 dpi. PrP^{C} expression in the CNS is essential for efficient transmission and neuropathology of the scrapie agent (Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997). As *CD21-cre*PrP$^{stop/-}$ mice have no PrP^{C} expression in the CNS, it is not anticipated that these animals could develop clinical TSE disease (Tuzi, Clarke et al. 2004). For this reason, no animals were left beyond 105 dpi. However to determine if this was the case, some transgenic animals were inoculated ic with the ME7 scrapie agent directly into the brain. Spleens were harvested from all time points and the cellular sites of PrP^{Sc} accumulation were determined. PrP^{Sc} has been shown to co-purify with TSE agent infectivity and is considered by many to be the sole component of the TSE infectious agent and therefore was used as a biochemical marker of disease in this study (Bolton, McKinley et al. 1982).

In animals with PrP^{C} expression switched on specifically in FDCs, the scrapie agent, as detected by PrP^{Sc} immunolabelling, located to the FDC network by 35 dpi. The deposition of PrP^{Sc} on the FDC increases at later time points, suggesting that the scrapie agent is efficiently replicating on the FDC networks. However, when PrP^{C} is switched on only in $CD21^{+}$ B lymphocytes, PrP^{Sc} is found only within the TBMs. These data show that PrP^{C} expressing FDCs are responsible for replicating the scrapie agent within the lymphoid tissue. Without PrP^{C} expression on FDCs the scrape agent was unable to replicate within the lymphoid tissues and is instead taken up by TBMs.

7.3 Results

7.3.1 Experimental design

To determine if PrP^{C} expression exclusively on FDCs is sufficient to support TSE agent replication in the lymphoid tissues, the *CD21-cre*$PrP^{stop/-}$ mouse line was injected ip with ME7 scrapie. Animals were aged to 8 weeks, lethally γ-irradiated and given donor bone marrow 24 h later as indicated. To restrict PrP^{C} expression specifically to FDCs, *CD21-cre*$PrP^{stop/-}$ mice were given bone marrow from Cre-negative littermates. However, other transgenic host/bone marrow combinations were also produced as control lines and these are summarised in Table 5.1. Animals were used in experiments at 100 d post bone-marrow reconstitution to allow for efficient replacement of host bone-marrow derived cells with donor counterparts. Mice from each group were then injected ip with 20 µl of a 1% (wt/vol) scrapie brain homogenate. Animals were then culled at 35, 70 and 105 days post-inoculation (dpi) and tissues were collected to assess the cellular sites of PrP^{Sc} accumulation in the spleen. As animals have no PrP^{C} expression in the CNS, no animals were expected to develop clinical disease. For this reason, no animals were left beyond the 105 dpi time point. However, to determine if this was true, some *CD21-cre*$PrP^{stop/-}$ animals were infected with scrapie directly into the CNS via ic injection alongside 129/Ola WT controls. A summary of experimental design can be found in Figure 7.1.

The cellular sites of PrP^{Sc} accumulation in the spleen were determined by immunolabelling for PrP. Immunohistochemical analysis was carried out using the

Host Genotype	Donor Genotype	PrP^C switched on	Nomenclature
CD21-cre$PrP^{stop/-}$	$PrP^{stop/-}$	FDCs only	Cre^{-ve} → *CD21-cre*$PrP^{stop/-}$
CD21-cre$PrP^{stop/-}$	*CD21-cre*$PrP^{stop/-}$	FDCs and B lymphocytes	Cre^{+ve} → *CD21-cre*$PrP^{stop/-}$
$PrP^{stop/-}$	*CD21-cre*$PrP^{stop/-}$	B lymphocytes only	Cre^{+ve} → $PrP^{stop/-}$
$PrP^{WT/-}$	$PrP^{WT/-}$		$PrP^{WT/-}$ → $PrP^{WT/-}$

Table 5.1 Summary of experimental lines used in scrapie experiments

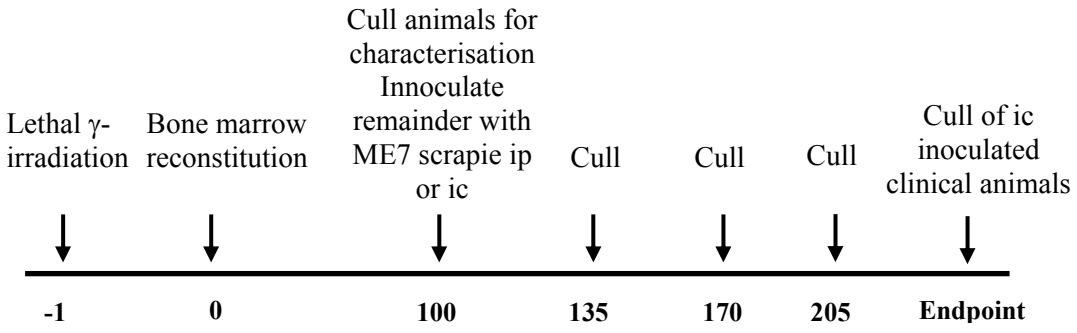


Figure 5.1 Summary of experimental design

rabbit anti-PrP PAb antibody 1B3 and confirmed using the mouse anti-PrP MAb 6H4. As both antibodies gave similar results, only immunohistochemistry images from 1B3 immunolabelling are shown. Treatment with PK digests the cellular PrP^{C} leaving the PK-resistant core of PrP^{Sc} intact (Manson, Clarke et al. 1994b). This property of PrP^{Sc} can be used to distinguish PrP^{C} and PrP^{Sc} on histological specimens by using the paraffin-embedded tissue (PET) blot method**.** This method was used to confirm if the disease specific PrP (PrP^{d}) detected by immunohistochemistry were scrapie-associated PrP^{Sc}. Detection of PrP^{d} and PrP^{Sc} have been confirmed by many studies to be reliable markers of the ME7 scrapie agent and in most cases correlate closely with presence of TSE agent infectivity (Bolton, McKinley et al. 1982; McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009).

7.3.2 Pathogenesis of ME7 scrapie at 35 dpi in mice with PrP^{C} expression exclusively on FDCs

Animals were culled at 35 dpi with the ME7 scrapie agent and spleens were harvested for analysis of the cellular sites of PrP^{Sc} accumulation. In spleens with PrP^{C} expression exclusively on Cre-expressing FDCs, and in $PrP^{WT/-}$ control spleens, accumulation of the abnormal, disease-associated PrP^{d} occurred in the germinal centres of the spleen (Figs 7.2). This PrP^{d} was located on the FDC network as shown by immunolabelling of serial sections for PrP^{d}, FDCs and B lymphocytes (Fig 7.3). A

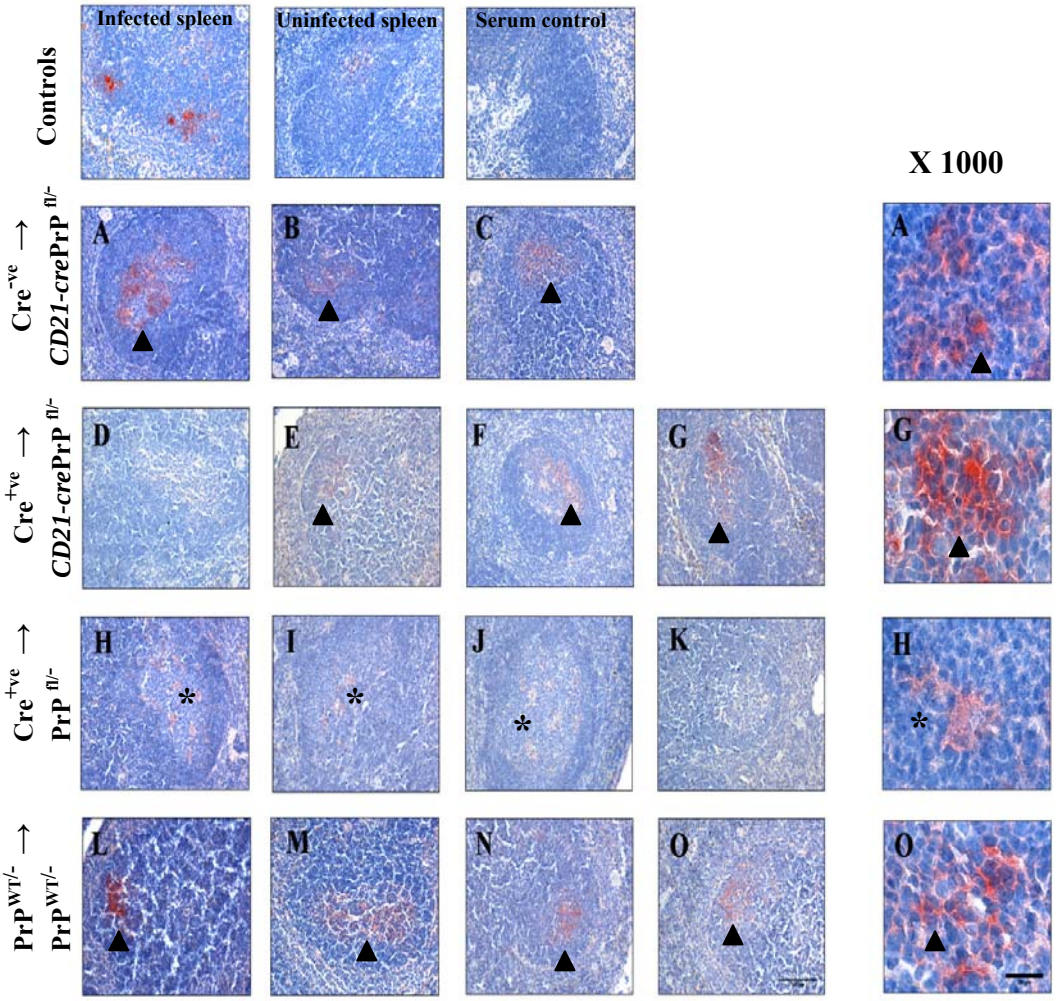


Figure 7.2 PrP^d immunolabelling in the spleen at 35 dpi with the ME7 scrapie agent

Four animals from each experimental line were culled at 35 dpi with the ME7 scrapie agent and spleens were immunolabelled to detect PrP^d (PAb 1B3, red). One animal was lost to an intrcurrent death.

All spleens with PrP^C expression exclusively in Cre-expressing FDCs (A-G) or $PrP^{WT/-}$ mice with PrP^C expressing FDCs (L-O), show PrP^d immunolabelling at low levels on the FDC networks (▲) in the follicles. However, spleens from animals with PrP^C-deficient FDCs (H-K) do not show immunolabelling on the FDC network. In these animals, the only positive PrP^d immunolabelling is found within the TBMs (*). These data demonstrate that the scrapie agent is able to localise to the follicles of the spleen independently of PrP^C expression as PrP^d is present on FDCs of animals expressing PrP^C exclusively on the FDCs. If FDCs are PrP^C deficient, the agent is localised to the follicle but is found mainly within the TBMs. Scale bar on main figure100 µm. Scale bar on x 1000 magnification images 20µm. Sections counterstained with haematoxylin, blue.

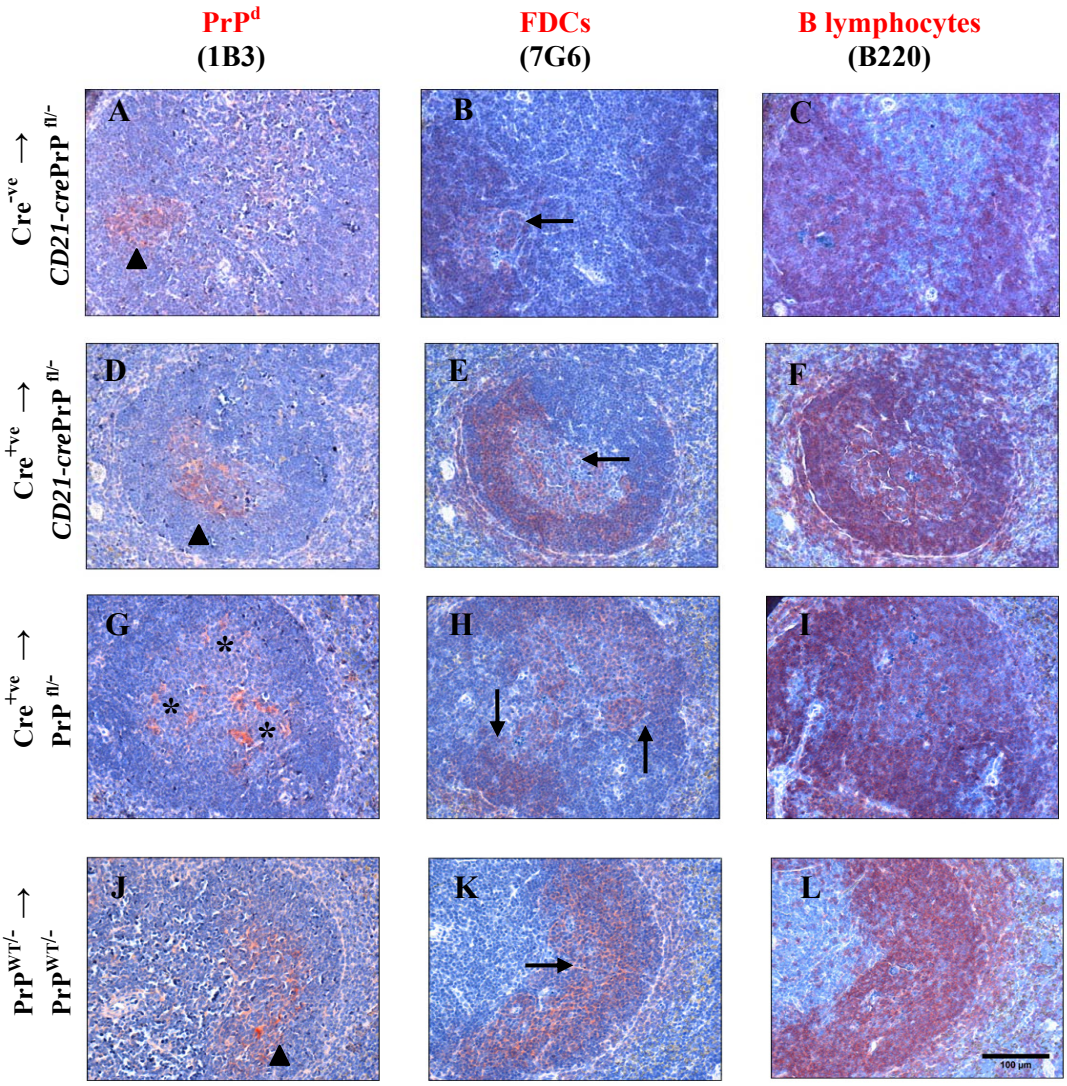


Fig 7.3 Immunohistochemical analysis of PrP^d, FDC and B lymphocytes in spleens of mice taken at 35 dpi with the ME7 scrapie agent
Serial sections from spleens were immunolabelled for PrP^d (PAb 1B3, red), FDCs and $CD21^+$ B lymphocytes (MAb 7G6, blue) and B lymphocytes (MAb B220, red) to determine if PrP^d detected was co-localised to the FDC network. One animal is shown from each experimental transgenic line.

In spleens with PrP^C expression exclusively on Cre-expressing FDCs (A-F), or with PrP^C-expressing FDCs in $PrP^{WT/-}$ mice (J-L), PrP^d (▲) is localised to the FDC networks (⟶) as shown in serial sections. PrP^d location in spleens with PrP^C deficient, FDCs (G-I) PrP^d was did not co-localise to the FDC networks but instead was found exclusively within TBMs (*) in the follicles. Scale bar 100 µm. Sections counterstained with haematoxylin, blue.

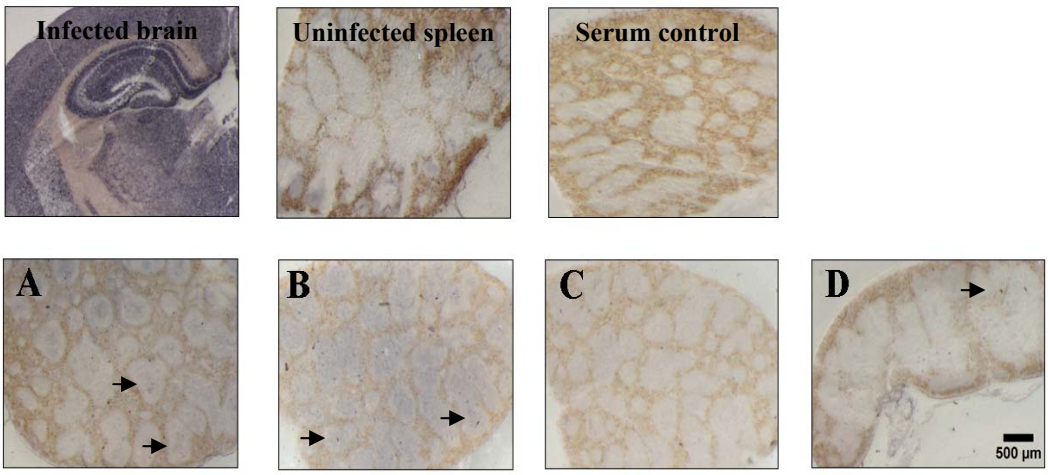


Figure 7.4 PET blot analysis of PrP^{Sc} accumulation in spleens taken at 35 dpi with the ME7 scrapie agent

Paraffin-embedded sections on nitrocellulose membranes were treated with PK to remove any native PrP^{C}, then immunolabelled (PAb, 1B3, blue/black) to detect any remaining PrP^{Sc}. One representative example from each transgenic line is shown. **A,** Cre^{-ve}→*CD21-cre*$PrP^{fl/-}$; **B,** Cre^{+ve}→*CD21-cre*$PrP^{fl/-}$; **C,** Cre^{+ve}→$PrP^{fl/-}$; **D,** $PrP^{fl/-}$→$PrP^{fl/-}$). Scale bar 500 µm.

Due to the low levels of PrP^{d} detected in infected tissue at this stage by immunohistochemistry (Fig 7.2), PrP^{Sc} is not readily detected after PK treatment in any of the transgenic experimental lines, however a few follicles in spleens with PrP^{C}-expressing FDCs have a little PrP^{d} labelling present (➔). Therefore PrP^{d} detected in these tissues by immunohistochemistry can be confirmed to be PrP^{Sc}.

little immunolabelling of PrP^d was still detected in the follicles after PK treatment, however, this was only at very low levels as animals are only expressing half copy number levels of *Prnp* and were culled early in the incubation period (Fig 7.4). These data suggest that in animals with PrP^C expression exclusively on FDCs at 35 dpi with the ME7 scrapie agent, the TSE agent can locate to the follicles of the spleen and begin to replicate on the PrP^C-expressing FDC networks.

In mice with PrP^C deficient FDCs, PrP^d was detected in the spleen at 35 dpi (Figs 7.2). However, the PrP^d detected did not co-localise to the FDC networks. Instead immunolabelling of PrP^d was located exclusively within TBMs within the follicle (Fig 7.3). Analysis of PET blots of spleens from these animals could not confirm the presence of PrP^{Sc} (Fig 7.4).

7.3.3 Pathogenesis of ME7 scrapie at 70 dpi in mice with PrP^C expression exclusively on FDCs

At 70 dpi with the ME7 scrapie agent, spleens with PrP^C expression exclusively on Cre-expressing FDCs, and in $PrP^{WT/-}$ control spleens, accumulation of the abnormal, disease-associated PrP^d occurred in the germinal centres of the spleen at a greater intensity than that seen in spleens from animals culled at 35 dpi (Figs 7.5). This PrP^d was located on the FDC network as shown by immunolabelling of serial sections for FDCs and B lymphocytes (Fig 7.6). Furthermore, the PrP^d accumulations detected upon the FDC networks were confirmed to be PK-resistant, scrapie agent-associated PrP^{Sc} using the PET blot method (Fig 7.7).

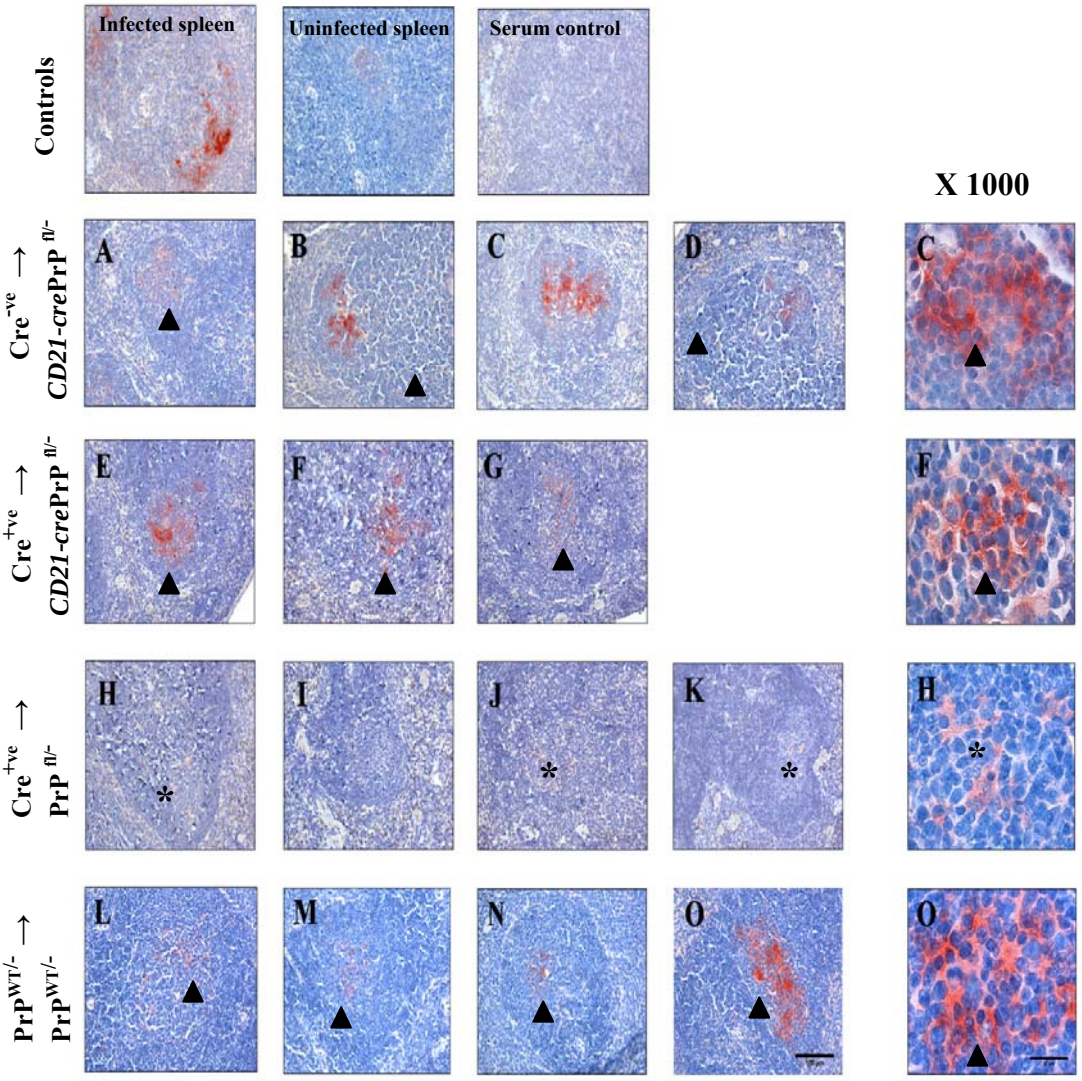


Figure 7.5 PrP^d immunolabelling in spleen at 70 dpi with the ME7 scrapie agent
Four animals from each experimental line were culled at 70 dpi with ME7 scrapie and spleens were immunolabelled to detect PrP^d (PAb 1B3, red).
All spleens with PrP^C expression exclusively in Cre-expressing FDCs (A-G) or $PrP^{WT/-}$ mice with PrP^C expressing FDCs (L-O), show PrP^d immunolabelling on the FDC networks (▲) within the follicles at a greater level than that seen in spleens from mice culled at 35 dpi. This indicates the scrapie agent is successfully replicating on the PrP^C-expressing FDCs despite all other cell types within the animal being *Prnp*$^{-/-}$ (A-D) or only $CD21^+$ B lymphocytes also expressing PrP^C (E-G).

Spleens from animals with PrP^C-deficient FDCs (H-K) do not show any immunolabelling of PrP^d on the FDC, instead, PrP^d is detected only within neighbouring TBMs (*). This suggests the scrapie agent is unable to replicate on PrP^C deficient FDCs and any PrP^d from innocula has been cleared from FDC networks and sequestered by TBMs in the follicle. Scale bar on main figure 100 μm. Scale bar on x 1000 magnification images 20μm. Sections counterstained with haematoxylin, blue.

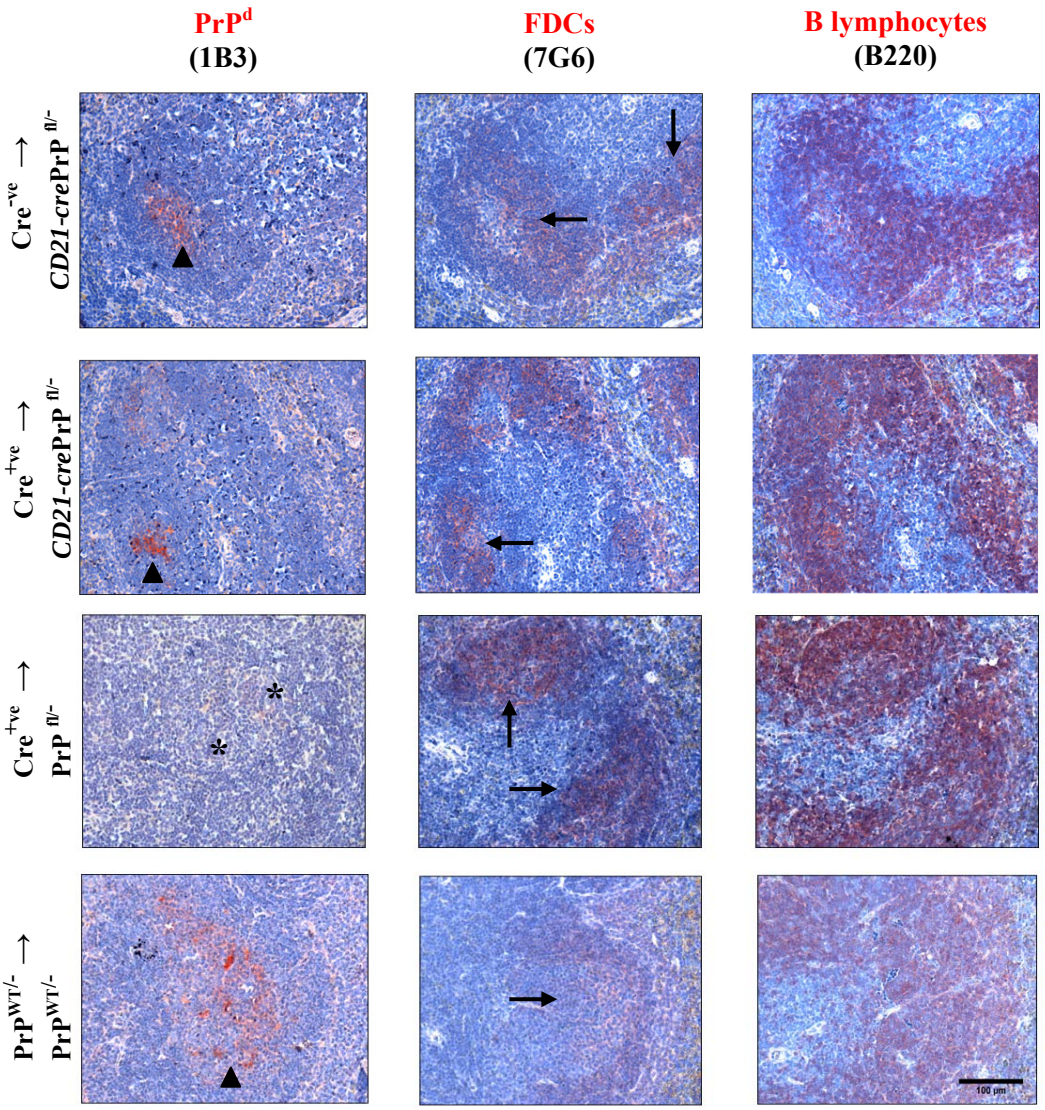


Fig 7.6 Immunohistochemical analysis of PrP^d, FDC and B lymphocytes in spleens of mice taken 70 dpi with the ME7 scrapie agent

Serial sections from spleens were immunolabelled for PrP^d (PAb 1B3, red), FDCs and $CD21^+$ B lymphocytes (MAb 7G6, red) and B lymphocytes (MAb B220, red) to determine if PrP^d detected co-localised to the FDC network. One animal is shown from each experimental transgenic line.

In spleens with PrP^C expression exclusively on Cre-expressing FDCs (A-F), or with PrP^C-expressing FDCs in $PrP^{WT/-}$ mice (J-L), PrP^d (▲) is localised to the FDC networks (⟶) as shown in serial sections. PrP^d in spleens with PrP^C deficient FDCs (G-I) was not present on the FDC networks but instead was found exclusively within TBMs (*) in the B lymphocyte follicles. Scale bar 100 µm. Sections counterstained with haematoxylin, blue.

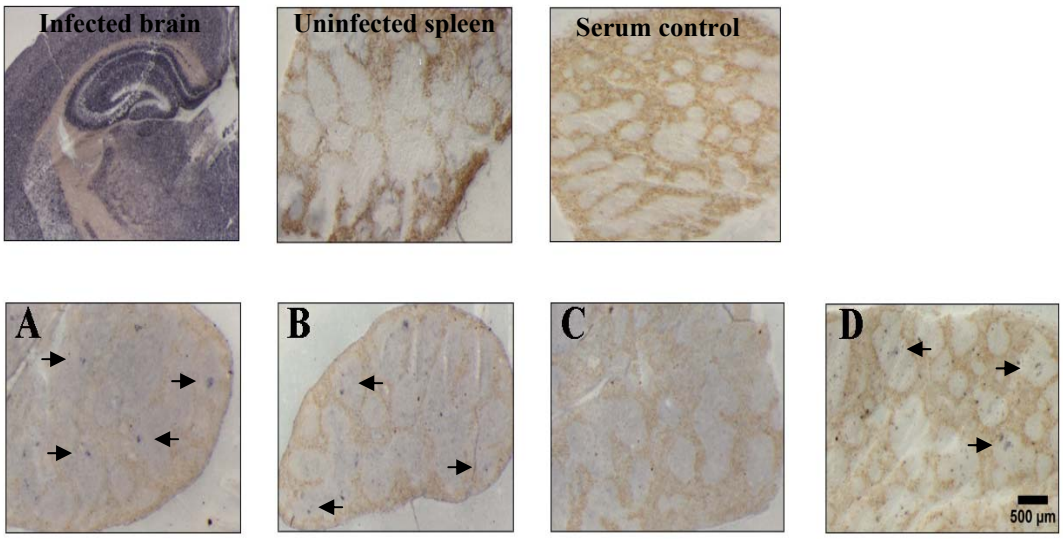


Figure 7.7 PET blot analysis of PrP^{Sc} accumulation in spleens taken at 70 dpi with the ME7 scrapie agent

Paraffin-embedded spleens sections on nitrocellulose membranes were treated with PK to remove any native PrP^{C} and then immunolabelled (PAb 1B3, blue) to detect any remaining PrP^{Sc}. One representative example from each transgenic line is shown. **A,** Cre^{-ve}→ *CD21-cre*$PrP^{fl/-}$; **B,** Cre^{+ve}→ *CD21-cre*$PrP^{fl/-}$; **C,** Cre^{+ve}→ $PrP^{fl/-}$; **D,** $PrP^{fl/-}$ → $PrP^{fl/-}$). Scale bar 500 µm

In spleens with PrP^{C} expression exclusively on Cre-expressing FDCs (A, B), or with PrP^{C}-expressing FDCs in $PrP^{WT/-}$ mice (D), PrP^{d} immunolabelling is present in the follicles after PK treatment (➔). Therefore PrP^{d} detected in these tissues by immunohistochemistry (Fig 7.5) can be confirmed to be the disease-associated PrP^{Sc}. In contrast, spleens from mice with PrP^{C} deficient FDCs (C) show no PrP^{Sc} in the follicles. This confirms that PrP^{C} expressing FDCs actively replicate the TSE agent in the lymphoid tissues.

From these data it can be concluded that PrP^C expression exclusively on FDCs is sufficient to support replication of the scrapie agent in peripheral lymphoid tissues, confirming that the FDCs actively replicate the TSE agent. Furthermore, no significant differences were observed in the levels of deposition of PrP^d on Cre-expressing, PrP^C-expressing FDCs in mice that received Cre^{+ve} or Cre^{-ve} bone marrow. This suggests that PrP^C expression on $CD21^+$ B lymphocytes is irrelevant to peripheral disease pathogenesis as previously reported (Klein, Frigg et al. 1998; Brown, Stewart et al. 1999; Mohan, Brown et al. 2004).

In spleens from mice with PrP^C deficient FDCs, no deposition of PrP^d was observed on the FDC networks. Instead, if PrP^d was detected at all, it was only found within TBMs (Fig 7.5). Immunolabelling of serial sections for FDCs and B lymphocytes confirmed that PrP^d immunolabelling did not co-localise with FDC networks (Fig 7.6). The levels of PrP^d observed within the TBMs were too low to be confirmed by the PET blot method as PrP^{Sc} (Fig 7.7). From these data it can be concluded that PrP^C-expressing FDCs are essential for the scrapie agent to accumulate within the lymphoid tissues. Without active replication of the agent by PrP^C expressing FDCs, the scrapie agent appears to be scavenged by TBMs in the follicle and possibly degraded. Furthermore, these animals with PrP^C deficient FDCs had been given Cre^{+ve} bone marrow so have PrP^C expression switched on exclusively in $CD21^+$ B lymphocytes. These data also confirm that PrP^C expression by mature B lymphocytes is not sufficient to support replication of the scrapie agent in the spleen as previously reported (Montrasio, Cozzio et al. 2001).

7.3.4 Pathogenesis of ME7 scrapie at 105 dpi in mice with PrP^C expression exclusively on FDCs

At 105 dpi with the ME7 scrapie agent in spleens with PrP^C expression exclusively on Cre-expressing FDCs, and in $PrP^{WT/-}$ control spleens, accumulation of the abnormal, disease-associated PrP^d occurred in the germinal centres of the spleen at a greater intensity than that seen in spleens from animals culled at 70 dpi (Fig 7.8). As previous, PrP^d was co-localised to the FDC network as shown by immunolabelling in serial sections for FDCs and B lymphocytes (Fig 7.9). Furthermore, PrP^d accumulation within the germinal centres was confirmed to be the PK-resistant, scrapie-associated PrP^{Sc} using the PET blot method (Fig 7.10). These results further supports data from previous time points showing that the PrP^C expression exclusively on FDCs is sufficient to support scrapie agent replication in the peripheral lymphoid tissues and confirms that PrP^C-expressing FDCs actively replicate the TSE agent in the lymphoid tissue. Additionally, no significant differences were seen in deposition of PrP^d in spleens from mice with PrP^C expression exclusively on Cre-expressing FDCs that received Cre^{+ve} or Cre^{-ve} bone marrow indicating that PrP^C expression on $CD21^+$ B lymphocytes is irrelevant to peripheral disease pathogenesis of the scrapie agent.

In spleens from mice with Cre-deficient, PrP^C deficient FDCs, there was no deposition of PrP^d on the FDC networks (Fig 7.8). Furthermore, levels of PrP^d detected within the TBMs at 105 dpi were greatly reduced in comparison to that seen at 35 and 70 dpi (Figs 7.2 and 7.5 respectively). Immunolabelling of serial sections for FDCs and B lymphocytes confirmed that the little PrP^d immunolabelling present

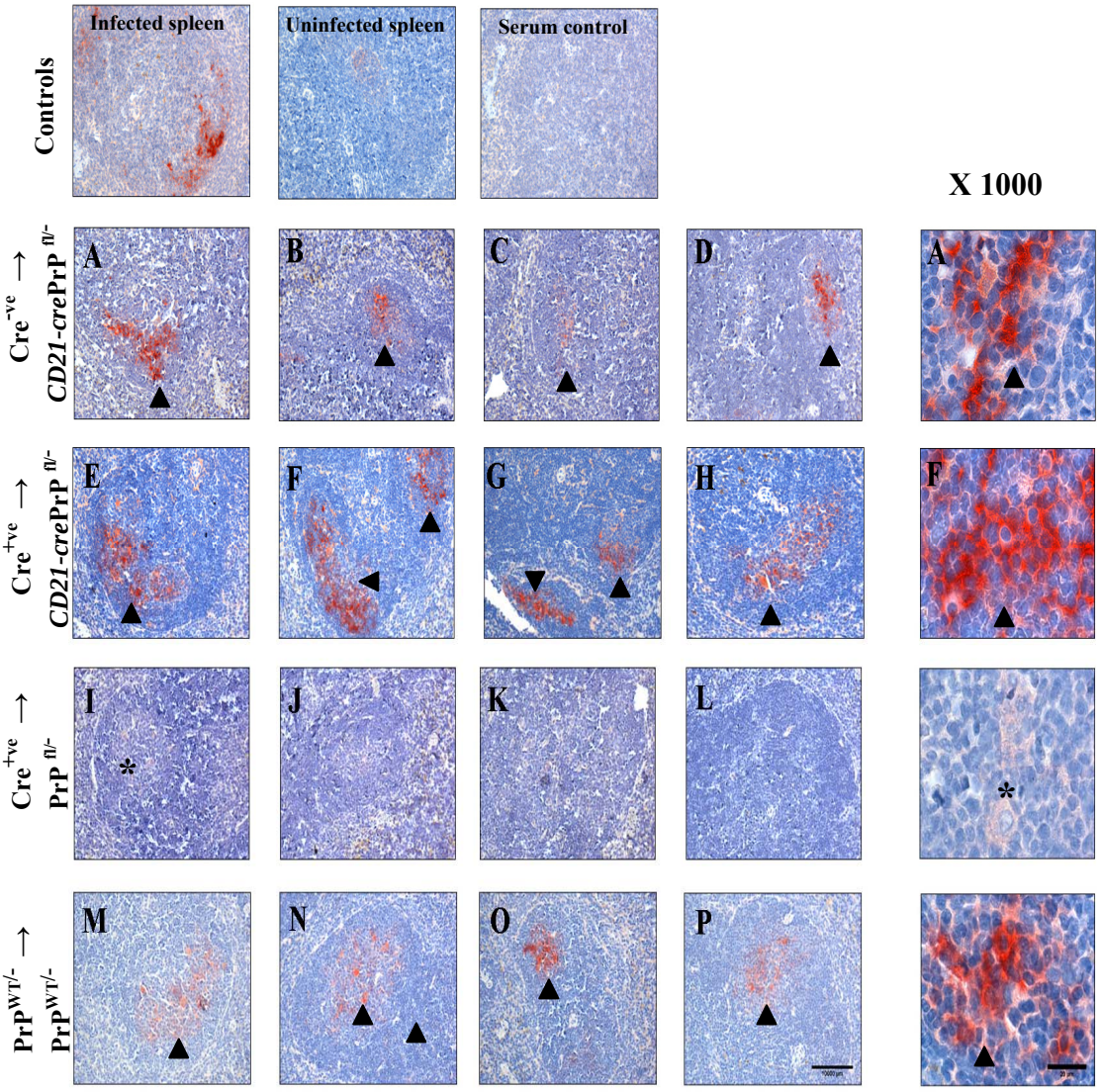


Figure 7.8 PrP^d immunolabelling in the spleen at 105 dpi with the ME7 scrapie agent

Four animals from each experimental line were culled at 105 dpi with the ME7 scrapie agent and spleens were immunolabelled to detect PrP^d (PAb 1B3, red).

All spleens with PrP^C expression exclusively in Cre-expressing FDCs (A-H) or $PrP^{WT/-}$ mice with PrP^C expressing FDCs (M-P), show PrP^d immunolabelling on the FDC networks (▲) within the follicles at an increased level than that seen in spleens from animals culled at 70 dpi. This indicates the scrapie agent is continuing to replicate on the PrP^C-expressing FDCs despite all other cell types within the animal being PrP^C-deficient (A-D) or only $CD21^+$ B lymphocytes additionally expressing PrP^C (E-H).

Spleens from mice with PrP^C deficient FDCs (I-L) show little/no immunolabelling of PrP^d at this time point. PrP^d is detected only within TBMs (*) in one animal. This suggests the scrapie agent is still unable to replicate on PrP^C deficient FDCs and as levels of PrP^d staining within TBMs are reduced in comparison to earlier time points (Fig 7.2 and 7.5), TBMs are most likely degrading the scrapie agent. Scale bar on main figure100 µm. Scale bar on x1000 magnification images 20µm. Sections counterstained with haematoxylin, blue.

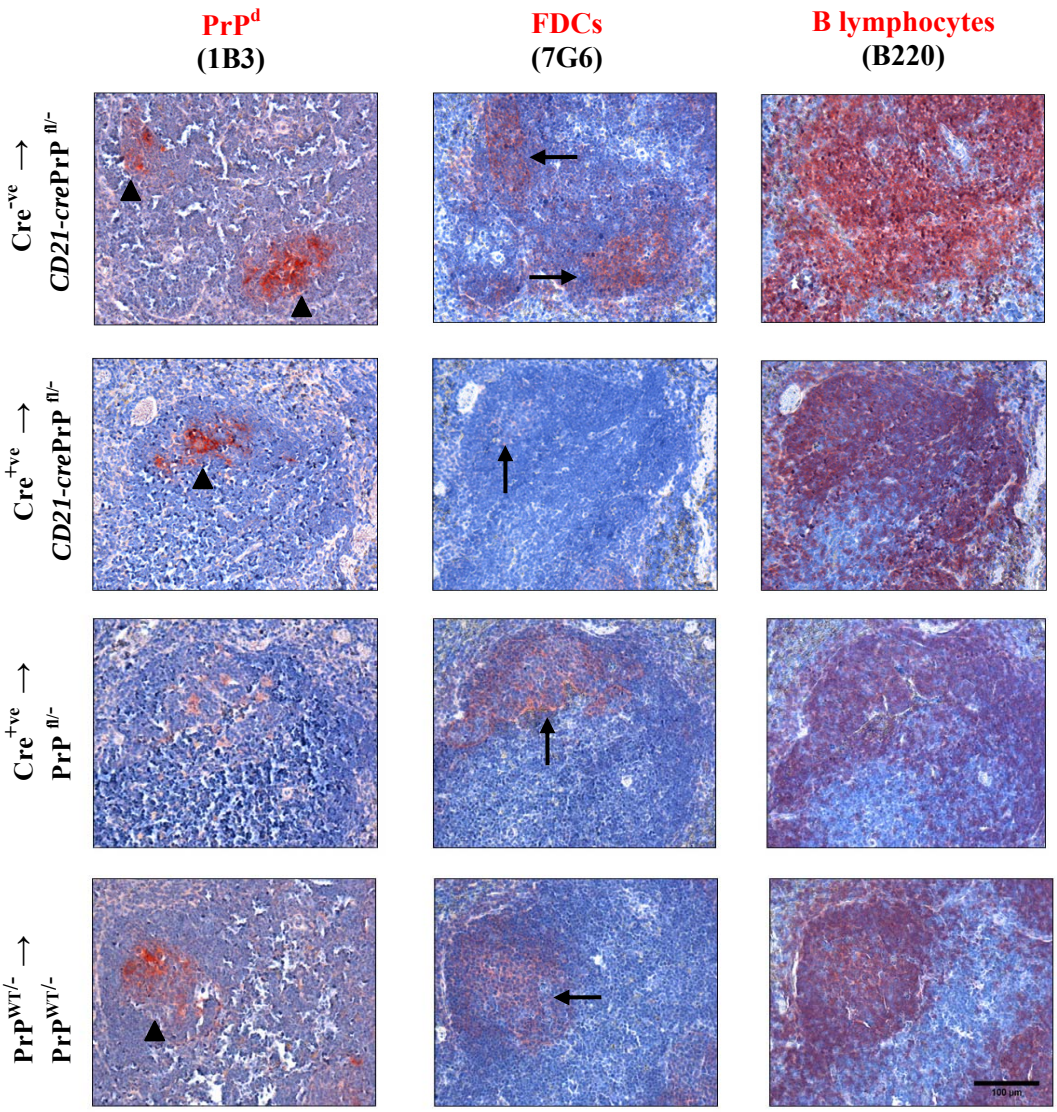


Fig 7.9 Immunohistochemical analysis of PrP^d, FDC and B lymphocytes in spleens of mice taken at 105 dpi with the ME7 scrapie agent

Serial sections from spleens were immunolabelled for PrP (PAb 1B3,red), FDCs and $CD21^+$ B lymphocytes (MAb 7G6, red) and B lymphocytes (MAb B220, red) to determine if PrP^d detected was co-localised to the FDC network. One animal is shown from each experimental transgenic line.

In spleens with PrP^C expression exclusively on Cre-expressing FDCs (A-F), or with PrP^C-expressing FDCs in $PrP^{WT/-}$ mice (J-L), PrP^d (▲) is localised to the FDC networks (⟶) as shown by serial sections. PrP^d in spleens with PrP^C deficient FDCs (G-I), was not present on the FDC networks but instead was found exclusively within TBMs (*) in the follicles. Scale bar 100 µm. Sections counterstained with haematoxylin, blue.

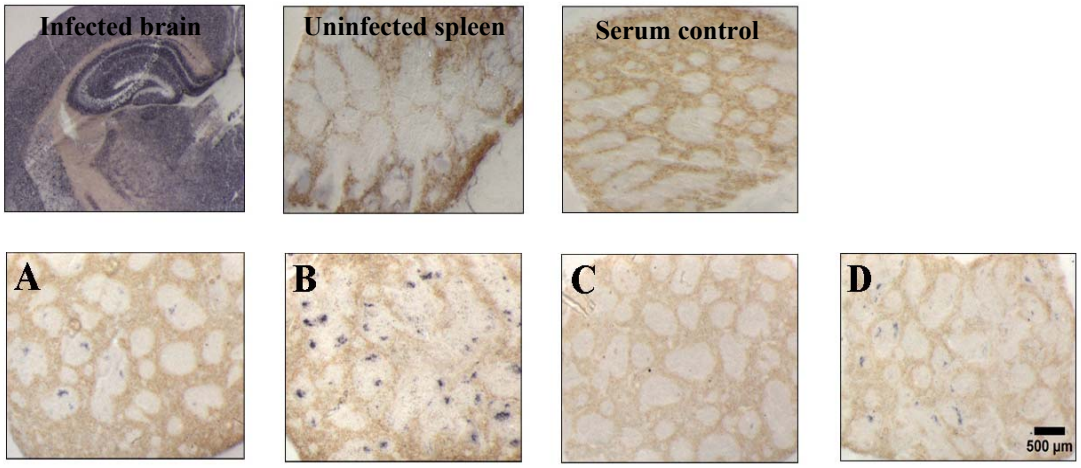


Figure 7.10 PET blot analysis of PrP^{Sc} accumulation in spleens taken at 105 dpi with the ME7 scrapie agent

Paraffin-embedded sections on nitrocellulose membranes were treated with PK to remove any native PrP^{C}, then immunolabelled (PAb 1B3, blue/black) to detect any remaining PrP^{Sc}. One representative example from each transgenic line is shown **A,** Cre^{-ve}→ *CD21-cre*$PrP^{fl/-}$; **B,** Cre^{+ve}→ *CD21-cre*$PrP^{fl/-}$; **C,** Cre^{+ve}→ $PrP^{fl/-}$; **D,** $PrP^{fl/-}$ → $PrP^{fl/-}$). Scale bar 500 µm.

In spleens with PrP^{C} expression exclusively on Cre-expressing FDCs (A, B), or with PrP^{C}-expressing FDCs in $PrP^{WT/-}$ mice (D), PrP^{d} immunolabelling is present in the follicles after PK treatment (➧). Therefore PrP^{d} detected in these tissues by immunohistochemistry (Fig 7.8) can be confirmed to be the disease-associated PrP^{Sc}. In contrast, spleens from mice with PrP^{C} deficient FDCs (C) show no PrP^{Sc} in the follicles. This confirms that PrP^{C} expressing FDCs actively replicate the TSE agent in the lymphoid tissues.

did not co-localise with FDC networks (Fig 7.9). The levels of PrP^{d} accumulation observed within the TBMs were too low to be confirmed by the PET blot method as PrP^{Sc} (Fig 7.10). These data confirm that PrP^{C}-expressing FDCs are essential for the scrapie agent to replicate within the lymphoid tissues and without active replication by FDCs, the scrapie agent is scavenged by neighbouring TBMs. As levels of PrP^{d} immunolabelling within the TBM are reduced over time it appears that the TBMs within the B lymphocyte follicle are degrading the scrapie agent.

7.3.5 TSE disease in the CNS of mice with PrP^{C} expression exclusively on FDCs

Previous studies have shown that host expression of PrP^{C} is absolutely required for TSE disease to occur. *CD21-cre*$PrP^{stop/-}$ animals only express PrP^{C} on FDCs and mature B lymphocytes and are therefore TSE disease should not be able to progress to the CNS and develop into clinical disease in these animals. For this reason, the 105 dpi time point after ME7 scrapie agent exposure was the final time point included in this experiment as no animals were expected to develop clinical disease. However to ensure that *CD21-cre*$PrP^{stop/-}$ mice are resistant to TSE disease in the CNS, some animals were injected with the ME7 scrapie agent directly into the CNS via ic injection alongside 129/Ola WT controls. WT animals developed TSE clinical disease with positive neuropathology in the brain with an average incubation period of 158 ± 2 dpi (Chapter 5 Fig 5.10). The classical hallmarks of TSE infection in the brain are deposition of abnormal, disease-associated PrP^{d}, gliosis and spongiform pathology (vacuolation). TSE disease in the brains of WT animals was confirmed by positive immunolabelling of PrP^{d}, activated astrocytes ($GFAP^{+}$ cells) or microglia ($IBA\text{-}1^{+}$ cells) in the brains of transgenic animals in comparison to WT controls

(Chapter 5 Fig 5.8). Furthermore, lesion profiling of the TSE specific vacuolation in specific areas of the brain is commonly used to characterise TSE disease in mice. Typical neuropathology, characteristic of infection with the ME7 scrapie agent was observed in the brains of control mice (Chapter 5 Fig 5.9). In contrast, *CD21-cre*PrP$^{stop/-}$ mice ic injected with ME7 scrapie are still alive at 313 dpi at point of writing. This exceeds the mean incubation time of 297 ± 4 dpi in *CD21-cre*PrP$^{fl/-}$ mice (Chapter 5 Fig 5.10), which have half copy level expression of *Prnp*. When surviving *CD21-cre*PrP$^{stop/-}$ mice are culled due to clinical disease or aging, brains and spleens will be harvested to determine if signs of scrapie pathology are present. However, due to lack of PrP^{C} expression in the CNS, it is not anticipated that these animals will develop clinical TSE disease.

7.4 Discussion

The *CD21-cre*PrP$^{stop/-}$ mouse line, with irradiation and reconstitution with non-Cre-expressing bone marrow, was previously shown to allow PrP^{C} expression to be switched on specifically on FDCs without adverse effects on FDC status or the lymphoid tissues (Chapter 6). In this chapter the *CD21-cre*PrP$^{stop/-}$ line was injected ip with the ME7 scrapie agent to determine if PrP^{C} expression exclusively on FDCs is sufficient to support scrapie agent replication within the lymphoid tissue and conclusively determine whether FDCs themselves actively replicate the scrapie agent. If FDCs themselves actively replicate the TSE agent, then PrP^{C} expression exclusively on the FDC network should be sufficient to support replication of scrapie in the lymphoid tissue.

Immunolabelling of PrP^{Sc} in the lymphoid tissue was used to assess the cellular localisation of the scrapie agent. PrP^{Sc} has previously been shown to co-purify with TSE agent infectivity in tissues from scrapie-affected animals and many studies have confirmed this to be a reliable marker of TSE disease (Bolton, McKinley et al. 1982; McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009). PrP^{Sc} and infectivity accumulates in the lymphoid tissues prior to detection in the CNS and deposition of PrP^{Sc} on the FDC network can be seen as early as 4 weeks post peripheral inoculation (Brown, Stewart et al. 1999). In this experiment, animals were culled at 35, 70 and 105 dpi to assess the early pathogenesis of ME7 scrapie in transgenic and control lines. As animals in this study are expressing only half copy number levels of *Prnp*,

less deposition of PrP^{Sc} would be expected on the FDC networks than seen in previous studies using *Prnp*$^{+/+}$ animals. No animals were left to progress beyond 105 dpi. Host expression of PrP^{C} has been shown to be essential for effective pathogenesis of the scrapie agent (Büeler, Aguzzi et al. 1993; Blattler, Brandner et al. 1997). Therefore, as animals are *Prnp*$^{-/-}$ except in cells where Cre is expressed (FDCs and/or $CD21^{+}$ B lymphocytes), it is unlikely that CNS disease would ever develop. However, to verify this, some *CD21-cre*$PrP^{stop/-}$ animals were also injected ic with the ME7 scrapie agent along with 129/Ola WT mice as controls.

Animals with Cre-expressing, *Prnp*$^{+/-}$ FDCs had detectable PrP^{Sc} on the FDCs of the spleen at 35 dpi. This accumulation intensified by 70 dpi and 105 dpi suggesting active replication of the scrapie agent on the PrP^{C}-expressing FDCs. This shows that PrP^{C} expression on FDCs alone is sufficient to support replication of the scrapie agent in the lymphoid tissues. This confirms previous studies which implied that that PrP^{C}-expressing FDCs actively replicate the scrapie agent in the lymphoid tissue (McBride, Eikelenboom et al. 1992; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Mabbott, McGovern et al. 2002; Mabbott, Young et al. 2003; Glaysher and Mabbott 2007; Raymond, Aucouturier et al. 2007; Brown, Wathne et al. 2009).

In animals with PrP^{C} switched on only in $CD21^{+}$ B lymphocytes, little to no PrP^{d} labelling is found in follicle at 35 dpi and by 70 and105 dpi, the only labelling detected is within the TBMs in the follicle. Some studies have suggested a role for B lymphocytes in replicating the scrapie agent in the lymphoid tissue (Klein, Frigg et al. 1997). However, others conclude that B lymphocyte involvement is indirect and is due to the dependence of the FDCs on B lymphocyte-derived signals to maintain a

mature and functioning FDC network (Klein, Frigg et al. 1998; Brown, Stewart et al. 1999). Data from the *CD21-cre*PrP$^{stop/-}$ study shows that PrP^{C} expression on CD21^{+} mature B lymphocytes alone is not sufficient to support scrapie replication. Furthermore, no differences were seen in pathogenesis of scrapie when PrP^{C} was expressed on FDCs alone or on both FDCs and CD21^{+} B lymphocytes. These data suggest that CD21^{+} B lymphocytes do not have an active role in the replication of the scrapie agent within the lymphoid tissue. This is in agreement with data from a previous study which found over-expression of PrP^{C} on B-lymphocytes only was not sufficient to support scrapie replication in the lymphoid tissue (Montrasio, Cozzio et al. 2001).

Transgenic animals that were inoculated ic with the ME7 scrapie agent have still not succumbed to clinical TSE disease 313 dpi with the ME7 scrapie agent at time of writing. Previous studies using *Prnp*$^{+/-}$ mice (Tew and Mandel 1979) and data from this thesis using of *CD21-cre*PrP$^{fl/-}$ animals (Chapter 5) has shown that following ic injection with the scrapie agent, the incubation period of *Prnp*$^{+/-}$ mice is approximately 300 dpi (Chapter 5 Fig 5.10). *CD21-cre*PrP$^{stop/-}$ mice in this study have progressed beyond this time point without showing signs of TSE disease so it appears that this line will be resistant to disease in the CNS as expected.

These data show that with no PrP^{C} present on the FDCs, the scrapie agent is unable to replicate on FDCs after initial localisation and appears to be rapidly cleared from the FDC network by TBMs in the follicle. TBMs are capable of taking up immune-complex coated portions of FDC membranes and therefore have the potential to remove the scrapie agent directly from the FDC surface (Szakal and Tew 1992;

Sandberg, Al-Doujaily et al. 2011). Additionally, previous publications have shown that depletion of macrophages prior to scrapie infection decreases scrapie incubation period and enhances replication in the lymphoid tissues further suggesting a role for macrophages in the degradation of the scrapie agent (Beringue, Demoy et al. 2000). These data further confirm that PrP^{C} expressing FDCs actively replicate the scrapie agent on their surface and without this replication, the scrapie agent is cleared from the FDC networks by TBMs.

CHAPTER 8

General Discussion

8. General discussion

8.1 Introduction

Accumulation of the TSE agent in the lymphoid tissue occurs in many cases of natural TSE disease. Many natural scrapie infections of sheep have accumulation of PrP^{Sc} in the spleen and lymphoid tissues of prior to CNS disease (van Keulen, Schreuder et al. 1996; Andreoletti, Berthon et al. 2000; Heggebo, Press et al. 2000; Heggebo, Press et al. 2002) and deer which have been naturally infected with CWD also show PrP^{d} and agent infectivity within the lymphoid tissues (Sigurdson, Williams et al. 1999; Fox, Jewell et al. 2006). Furthermore, patients with vCJD show PrP^{d} and/or infectivity in the appendix and tonsils (Hilton, Fathers et al. 1998; Hill, Butterworth et al. 1999; Bruce, McConnell et al. 2001) and recent evidence has shown transmission of vCJD from contaminated blood products (Llewelyn, Hewitt et al. 2004; Peden, Head et al. 2004; Wroe, Pal et al. 2006; Peden, McCardle et al. 2010). These data, along with the current lack of available treatments and invariable fatality of infected patients, highlight the need to understand the pathogenesis of TSE disease within the lymphoid tissues to determine cellular targets for focused research into prophylactic treatments.

Previous studies investigating the role of the lymphoid tissues in TSE disease have highlighted PrP^{C}-expressing FDCs as an essential requirement for successful pathogenesis of the TSE agent within peripheral lymphoid tissues (McBride, Eikelenboom et al. 1992; Klein, Frigg et al. 1998; Brown, Stewart et al. 1999; Mabbott, Mackay et al. 2000b; Kaeser, Klein et al. 2001; Mabbott and Bruce 2002; Mabbott, McGovern et al. 2002; Prinz, Montrasio et al. 2002; Mabbott, Young et al.

2003; Prinz, Heikenwalder et al. 2003; Glaysher and Mabbott 2007; Raymond and Mabbott 2007; von Poser-Klein, Flechsig et al. 2008; Brown, Wathne et al. 2009). However, in models used so far, there has been no definitive way to exclusively examine the role of PrP^C-expressing FDCs in isolation from all other stromal, neural and lymphoid cells within the lymphoid tissue. One of the main functions of FDCs is to pick up antigenic protein complexes and retain them on their surface for weeks, months or even years, which is an essential component in the affinity maturation of B lymphocytes and the development of immunological memory (Tew and Mandel 1979; Gray and Skarvall 1988; Gray and Matzinger 1991; Ahmed and Gray 1996). Therefore, it is possible that the PrP^d observed on the FDC networks of TSE-affected animals is simply due to accumulation of the TSE agent on the FDC surface after replication on another cell type. An FDC-specific model was required to definitively determine whether FDCs themselves actively replicate the TSE agent or if they simply accumulate the agent after replication on another cell type. If FDCs are simply acquiring the TSE agent after replication on another cell type, investigations into which cell is responsible for replicating the TSE agent could provide a new therapeutic target for the prevention or prophylactic treatment of TSE disease.

The aims of this thesis were to create and characterise transgenic mouse models that allow the manipulation of PrP^C expression specifically on FDCs and subsequently to infect these animals with scrapie to determine the specific role of the FDCs in TSE pathogenesis within the lymphoid tissue. The hypothesis was explored that if FDCs are simply accumulating the TSE agent on their surface after replication on another cell type, the accumulation of the TSE agent will occur on PrP^C deficient FDCs when all other cell types within the lymphoid tissue retain PrP^C expression. If however, the

FDCs actively replicate the TSE agent in the lymphoid tissue, accumulation of PrP^{d} will not occur on the PrP^{C} deficient FDCs. Additionally, if FDCs are responsible for replicating the TSE agent within the lymphoid tissue, then PrP^{C} expression exclusively on FDCs should be sufficient to allow replication of the TSE agent within the lymphoid tissue

8.2 Critical analysis of the transgenic mouse models used in this study

The creation and characterisation of the FDC-specific transgenic mouse models were a crucial component of this study. Transgenic lines were extensively characterised before use in ME7 infection studies, however some important points regarding conditional gene targeting mice should be noted. The *ROSA26* mouse line is a reporter strain which is commonly used to characterise Cre-expression under cell specific promoters (Mao, Fujiwara et al. 1999). Promoter specific activation of Cre induces expression of β-galactosidase within Cre-expressing cells, which can subsequently be detected histologically using an X-gal stain. However, when analysing results achieved from such reporter strains, it is important to note that individual alleles may have a variable sensitivity to Cre activity as reported by Schmidt-Supprian and Rajewsky (Schmidt-Supprian and Rajewsky 2007). Therefore, the pattern of Cre-mediated DNA recombination will only be identical to that seen in reporter strains if the floxed alleles in compound transgenic mice have the same sensitivity to Cre as those in the reporter strain. In this thesis, the *ROSA26* reporter strain was used to provide an initial indication of the cellular locations of Cre-expression, however extensive characterisation was also carried out on the compound transgenic lines achieved by crossing *CD21-cre* mice with the *Prnp*-floxed mouse lines.

Cre toxicity is a phenomenon where transgenic expression of Cre is lethal to the cell due to DNA damage and or chromosomal abnormalities. This can occur due to the presence of cryptic or pseudo *LoxP* sites that are contained within the mouse genome undergoing Cre-mediated DNA recombination (Thyagarajan, Guimarães et al. 2000; Schmidt-Supprian and Rajewsky 2007; Semprini, Troup et al. 2007). Characterisation of the number of $CD21^+$ B lymphocytes (Chapter 3), the lymphoid tissue composition and microarchitecture, and the status and function of the FDCs (Chapters 4 and 6) all showed no apparent effects of toxicity in the Cre-expressing cells.

In the transgenic mouse lines used in this thesis, Cre is inserted after the *Cr2* promoter, which encodes CD21. CD21 was considered to be restricted to FDCs and mature B lymphocytes. (Reynes 1985; Liu 1997; Takahashi, Kozono et al. 1997; Heggebo, Press et al. 2002). However, in humans, expression of CD21 has also been reported on a subpopulation of immature thymocytes (Tsoukas and Lambris 1988; Wagner and Hansch 2006), peripheral T lymphocytes (Fox, Jewell et al. 2006; Peden, McCardle et al. 2010) and on human cervical epithelium (Sixbey, Lemon et al. 1986) . Within the mouse, expression has also been reported $CD4^+$ T lymphocytes found within the MLN, activated granulocytes and mucosal mast cells (Gray and Skarvall 1988; Gray and Matzinger 1991; Andrasfalvy, Prechl et al. 2002; Heggebo, Gonzalez et al. 2003; Llewelyn, Hewitt et al. 2004). These data indicate that expression of CD21 is more widespread than previously thought. However, all other cells currently reported to express CD21 are derived from the blood. Therefore lethal γ-irradiation and reconstitution with non-Cre expressing bone marrow should still restrict Cre expression to the FDC network.

The *CD21-creROSA26* mouse demonstrated that Cre was efficiently expressed in FDCs and mature B lymphocytes and that irradiation and reconstitution with WT bone marrow successfully restricted Cre expression to the FDC networks (Chapter 3). This was a key factor in using the *CD21-cre* mouse line for FDC specific manipulation of *Prnp* expression. Characterisation of both the *CD21-cre*PrP$^{fl/-}$ and the *CD21-cre*PrP$^{stop/-}$ lines confirmed the specificity and efficiency of the *CD21-cre* model by demonstrating efficient removal or expression of PrP^{C} specifically on the FDC networks (Chapters 4 and 6 respectively) which is in agreement with previous data published using this mouse line showing efficient Cre-mediated DNA recombination in mature B lymphocytes and FDCs (Kraus, Alimzhanov et al. 2004; Victoratos, Lagnel et al. 2006). Previous studies using the CD21-cre line to restrict Cre expression to the FDC network analysed DNA from enriched FDC populations from the spleen and used PCR to assess the DNA recombination in the FDCs (Victoratos, Lagnel et al. 2006). These experiments were not undertaken in this thesis. However, PCR analysis of whole blood, spleen and tail DNA sufficiently showed the correct chimerism between donor bone marrow and host Cre expression status.

A population of Cre-expressing neuronal or glial cells within the ganglia of both the submucosal and myenteric plexi of the intestine and in the brain were also detected (Chapter 3). No literature has reported expression of the CD21 protein within cells of these tissues as of yet. Furthermore, data from the bioGPS database which stores microarray data of gene expression profiles from various mouse and human tissues shows no non-lymphoid expression of *Cr2*; including samples from intestine and brain (Chapter 1, Fig 1.3 http://biogps.gnf.org) . Due to time constraints, it was not

possible to further investigate within which cell population Cre was activated in these tissues. However, the route of inoculation was adapted from oral to ip to take any Cre-mediated manipulation of *Prnp* in the intestine into consideration. In agreement with *CD21-creROSA26* characterisation studies in this thesis, Cre activation in the forebrain of the *CD21-cre* mouse line has recently been reported (Schmidt-Supprian and Rajewsky 2007). Cre activation in the forebrain has no effect on the early stages of disease incubation that were examined in the ME7 infection studies but a possible effect of Cre-activation in the brain was considered when analysing clinical animal data in this thesis.

The *Prnp* floxed mouse lines used were created via gene targeting using bacterial artificial chromosomes, which helps to prevent the problems of ectopic- and over-expression of transgenes and disruption to gene function where the transgene has been inserted (Tuzi, Clarke et al. 2004). The $PrP^{fl/-}$ model has been used with previous success to switch off PrP^{C} expression exclusively on Schwann cells in the peripheral nerves to determine their role in TSE agent neuroinvasion and achieved a 90% reduction of PrP^{C} levels (Bradford, Tuzi et al. 2009). Western blot quantification of PrP^{C} levels expressed was not carried out in this thesis. However, PCR analysis confirmed recombination of the floxed DNA and quantification of the co-localisation of PrP^{C} on immunohistochemical images showed efficient switching off/on of PrP^{C} on FDC in both mouse lines characterised (Chapters 4 and 6).

The compound transgenic mouse lines used in the ME7 scrapie agent infection studies only expressed half copy number levels of *Prnp* due to complications in breeding the *CD21-cre*$PrP^{fl/-}$line and the $Prnp^{stop}$ allele being lethal at homozygosity, as discussed

in Chapters 4 and 6, respectively. Previous studies have shown that expression levels of PrP^C inversely correlate with incubation periods of TSE infection in mice. Over expression of PrP^C leads to significantly shorter incubation periods (Fischer, Rulicke et al. 1996), whereas half copy number levels of *Prnp* expression extend the incubation period of disease (Manson, Clarke et al. 1994b). Therefore in the models used in this thesis, although disease incubation period was increased there should be no other differences in disease pathogenesis.

In summary, characterisation of the *CD21-creROSA26*, *CD21-cre*$PrP^{fl/-}$ and *CD21-cre*$PrP^{stop/-}$ mouse lines used in this thesis has sufficiently demonstrated gene manipulation specifically on FDCs after irradiation and transfer of WT bone marrow. Furthermore, no adverse effects were observed on the FDC status or general microarchitecture of the lymphoid tissue. Data from characterisation studies demonstrate that the *CD21-cre*$PrP^{fl/-}$ and *CD21-cre*$PrP^{stop/-}$ mice are robust *in vivo* models for manipulating expression of *Prnp* exclusively on FDCs.

8.3 Do FDCs express PrP^C?

The presence of PrP^C-expressing FDCs has been shown by many studies to be necessary for successful replication of the TSE agent (Brown, Stewart et al. 1999; Mabbott, Williams et al. 2000a; Mabbott, Mackay et al. 2000b; Mabbott, McGovern et al. 2002; Mabbott, Young et al. 2003; Mohan, Brown et al. 2004; Ierna, Farquhar et al. 2006; Brown, Wathne et al. 2009). However, it has not been confirmed that FDCs themselves produce the PrP^C protein that they express on their surface. It is important to determine if FDCs acquire or produce expression of PrP^C as the mechanisms

involved in this process could have implications on the deposition of PrP^{d} seen on the FDC surface in scrapie-affected animals.

One of the main roles of the FDC is to trap antigenic protein complexes which they can retain on their surface for long periods of time via expression of complement and Fc receptors (Fang, Xu et al. 1998; Prodeus, Goerg et al. 1998; Wu, Jiang et al. 2000). This is considered essential for affinity maturation of B lymphocytes and the development of immunological memory (Gray and Skarvall 1988; Gray, Kosco et al. 1991; Gray and Matzinger 1991). Furthermore, previous studies have shown that FDCs can acquire proteins, such as MHC class II, on their surface that they themselves do not express (Gray, Kosco et al. 1991; Thery, Regnault et al. 1999; Denzer, van Eijk et al. 2000). For these reasons, it is possible that FDCs acquire expression of PrP^{C} rather than producing this protein themselves.

Exosomal transport of membrane proteins is a mechanism cells can use to acquire proteins that they themselves do not express. Exosomes are membrane vesicles of 40-100 nm in diameter, which preferentially contain lipid rafts and GPI-anchored proteins (Stoorvogel, Kleijmeer et al. 2002). This makes exosomal transport a potential mechanism for the transportation of PrP^{C} protein which is also GPI-anchored. Neighbouring B lymphocytes in the follicle also express PrP^{C} and are a possible candidate for the transfer of PrP^{C} to FDCs (Mabbott, Brown et al. 1997). Indeed, previous studies have shown that FDCs can obtain proteins via exosomes from B lymphocytes (Denzer, van Eijk et al. 2000) but also from DCs (Thery, Regnault et al. 1999) and macrophages (Wang, Zhou et al. 2010). Furthermore, in vitro studies have shown that cells can release both PrP^{C} and PrP^{Sc} on exosomes and

have implicated this mechanism may be involved in the intracellular transfer of the TSE agent (Fevrier, Vilette et al. 2004; Fevrier, Vilette et al. 2005).

However, there is also evidence to support the expression of PrP^C by FDCs themselves. Studies analysing the development of FDC networks in SCID mice (Bruce, Brown et al. 2000) and neonatal mice (Ierna, Farquhar et al. 2006) found that PrP^C was detectable in the follicle at the same time as the development of mature FDC networks, suggesting that FDCs themselves express PrP^C. Additionally, studies using bone marrow chimeric mice which had mismatches in PrP^C expression between stromal and lymphoid components showed that PrP^C was detected upon FDCs by immunohistochemistry when the donor bone marrow was PrP^C deficient. This eliminates the possibility that bone marrow-derived cells are donating PrP^C expression to the FDCs in this model (Brown, Stewart et al. 1999). Furthermore, data from the microarray analysis of gene expression by enriched splenic FDCs show that *Prnp* is expressed in these cells indicating that they subsequently express the PrP^C protein (Huber, Thielen et al. 2005; Wilke, Steinhauser et al. 2010). Data from this thesis supports FDCs producing and expressing PrP^C protein themselves. When PrP^C was switched off exclusively on FDCs, little to no PrP^C was detected on the FDC network even though all neighbouring cells in the follicle retained PrP^C expression (Chapter 4, Figs 4.5 and 4.6). From this evidence, it appears that FDCs actively produce the PrP^C protein they express. Hypothetically, it may be possible for FDCs to also acquire PrP^C protein from other cell types. However the model used in this study where PrP^C expression is switched off exclusively on FDCs suggest that little to no PrP^C is acquired by the FDCs from other cell types.

8.4 The function of PrP^C in the immune sytem

The function of PrP^C is as of yet not fully understood, and many possible functions of this protein have been suggested. $PrP^{-/-}$ mice have no overt neurological phenotype although small changes in synaptic transmission (Collinge, Whittington et al. 1994; Colling, Collinge et al. 1996; Mallucci, Ratte et al. 2002), circadian rhythms (Tobler, Gaus et al. 1996), cognition (Coitinho, Roesler et al. 2003) and seizure thresholds (Walz, Amaral et al. 1999) have been reported. Additional suggested functions of PrP^C in neurones are copper binding (Hornshaw, McDermott et al. 1995; Jackson, Murray et al. 2001), cell signalling (Mouillet-Richard, Ermonval et al. 2000; Spielhaupter and Schätzl 2001) and both pro- (Paitel, Alves da Costa et al. 2002) and anti- (Kuwahara, Takeuchi et al. 1999; Bounhar, Zhang et al. 2001; Chiarini, Freitas et al. 2002) apoptotic activity. However, these proposed functions of PrP^C within the CNS do not always have bearing on what PrP^C function in the immune system, and more specifically on the FDCs, truly is. Maturation of DCs and monocytes has been reported to up-regulate PrP^C expression (Dürig, Giese et al. 2000; Burthem, Urban et al. 2001; Ballerini, Gourdain et al. 2006), whereas down-regulation has been reported upon activation of B and T lymphocytes in mice (Kubosaki, Yusa et al. 2001; Liu, Li et al. 2001). PrP^C is up-regulated in some functionally differentiated lymphocytes including a population of regulatory T lymphocytes (Huehn, Siegmund et al. 2004) and in memory CD8 T cells (Li, Liu et al. 2001; Goldrath, Luckey et al. 2004). Immune function in $PrP^{-/-}$ mice has not been addressed to any great extent. Studies carried out so far have shown PrP^C deficiency has no impact on expression levels of both MHC Class I and II, maturation of DCs, and numbers of $CD4^+$ and $CD8^+$ T lymphocytes and B lymphocytes are no different to that seen in WT counterparts

(Bueler, Fischer et al. 1992; Kubosaki, Yusa et al. 2001; Ballerini, Gourdain et al. 2006; Zhang, Steele et al. 2006). Recent studies have shown that PrP^C deficient T lymphocytes are more susceptible to oxidative stress (Aude-Garcia, Villiers et al. 2011) which is in agreement with previous publications demonstrating a neuroprotective role against oxidative stress for PrP^C expression by neurones (Mitteregger, Vosko et al. 2007). However, there is a lack of *in vivo* studies investigating the role of PrP^C during an active immune response, effects of PrP^C deficiency on the germinal centre or the role PrP^C may play specifically on FDCs. If PrP^C plays a protective role against oxidative stress or anti-apoptotic functions as discussed, FDC may express high levels of PrP^C due to their functional attributes, for example accumulating large amounts of protein on their surface.

Data from this thesis has shown that removing PrP^C specifically on FDCs had no effect on the area or number of FDC networks in the spleen (Chapter 4, Fig 4.4). In mice with PrP^C deficient FDCs, no observed difference was seen in expression of common FDC markers such as the complement receptors CD21/35 and bound complement C4 (Chapter 4, Fig 4.3) or on the ability of FDCs to capture immune complexes (Chapter 4, Fig 4.8) which suggests that PrP^C function does not impact on these processes. However, these observations are made during steady state conditions where animals are housed in individually ventilated cages in specified pathogen-free conditions. Perhaps an effect of PrP^C deficient FDCs would only become apparent after immunisation during initiation of a germinal centre response, or during an ongoing immune response. Furthermore, ultrastructural studies of PrP^C deficient FDCs in comparison to WT controls may also provide indications as to the function of PrP^C on the FDCs.

8.5 The role of FDCs in TSE pathogenesis

It has now been known for some time that many peripherally acquired TSE infections have a stage of replication in the lymphoid tissue prior to neuroinvasion and CNS disease. Studies investigating which cell type was responsible for replicating the agent have gradually led to the FDC being identified as the main candidate to replicate the TSE agent in the lymphoid tissue. Initial studies identified the stromal component of the spleen as having more TSE agent infectivity than that found in the pulp (Clarke and Kimberlin 1984). Subsequently, it was discovered that ionising radiation had no effect on scrapie pathogenesis indicating that the cell type responsible for replicating the agent was long lived and radio-resistant (Fraser and Farquhar 1987). As investigations into the cell type responsible for replicating the TSE agent continued, the involvement of T lymphocytes (Fraser and Dickinson 1978; Klein, Frigg et al. 1997), B lymphocytes (Klein, Frigg et al. 1998; Montrasio, Cozzio et al. 2001) or macrophages (Carp and Callaghan 1982; Beringue, Demoy et al. 2000) in actively replicating the TSE agent became more unlikely. Many studies showed that SCID mice, which lack T and B lymphocytes and FDCs, were resistant to peripherally acquired TSE infection (O'Rourke, Huff et al. 1994; Fraser, Brown et al. 1996; Lasmezas, Cesbron et al. 1996). As a role for T and B lymphocytes seemed unlikely these studies suggested that FDCs may be responsible for replicating the TSE agent.

FDCs had been shown to have relatively large quantities of PrP^C on their surface. Furthermore, immunohistochemical analysis of TSE-infected spleen showed high

levels of deposition of PrP^d on the FDC surface, which electron microscopy studies confirmed to be located to the FDC plasmalemma (Kitamoto, Muramoto et al. 1991; McBride, Eikelenboom et al. 1992; Brown, Ritchie et al. 2000; Jeffrey, McGovern et al. 2000). Animals with genetic deficiencies in TNFα (Mabbott, Williams et al. 2000a) or LTβ (Oldstone, Race et al. 2002), which have no mature FDC networks, were resistant to peripherally inoculated scrapie infection. Additionally, studies which temporarily dedifferentiated the FDC networks of mice prior to peripheral inoculation with the scrapie agent using the TNF-R1 homologue (Mabbott, McGovern et al. 2002) the LTβR-Ig fusion protein (Montrasio 2000; Mabbott, Mackay et al. 2000b) prevented or delayed clinical scrapie disease. These studies provided strong evidence that FDCs were responsible for replicating the TSE agent. However, in all of the above models, in addition to removing the PrP^C expressing FDC networks, more general effects on the immune system were also incited, such as impairment of macrophage function (Klein, Frigg et al. 1997; Mabbott, Williams et al. 2000a; Mabbott, McGovern et al. 2002) or removal of FDC networks resulting in no germinal centres (Montrasio 2000; Mabbott, Williams et al. 2000a; Mabbott, McGovern et al. 2002; Oldstone, Race et al. 2002) . Therefore it is possible that the effects on TSE pathogenesis could have been due to a more general immunodeficiency and not just the lack of FDCs. Bone marrow chimeric models, which used lethal γ- irradiation and reconstitution with donor bone marrow to create mismatches in PrP^C expression status between stromal and bone marrow-derived cells, showed that PrP^C expressing FDCs were essential for successful replication of scrapie whereas PrP^C expression on lymphocytes was irrelevant (Brown, Stewart et al. 1999). These models did not have a general effect on the immune system as was seen in previous models. However, PrP^C expression was retained by other neural, stromal

and epithelial cell types alongside the FDCs. For this reason, the possibility still remained that FDCs simply acquired the scrapie agent after replication on another cell type. An FDC-specific model, which had no impact on normal immune function, was required to definitively determine if FDCs simply acquire the TSE agent rather than actively replicate the agent themselves.

In this thesis, mouse models were created where PrP^C could be switched off (Chapter 4) or on (Chapter 6) exclusively on FDCs. Characterisation of these models determined the specificity and efficiency of Cre-mediated recombination of the floxed *Prnp* genes. Additionally we showed that there were no observed effects on the status or function of the FDC networks and no effects on lymphoid tissue microarchitecture. In animals in which PrP^C was switched off exclusively on FDCs that were inoculated ip with the ME7 scrapie agent, the scrapie agent initially localised to the FDC networks, but at later time points was only detectable within the neighbouring TBMs (Chapter 5). This was in contrast to animals which retained PrP^C expression on FDCs which showed PrP^d accumulation on the FDC network increasing over time. Furthermore, clinically scrapie-affected animals with PrP^C deficient FDC that had been injected directly into the CNS also showed no PrP^d accumulation on the FDC networks. Again, the only PrP^d detected in these animals was found within the TBMs. PrP^C deficient FDCs remained free of PrP^d accumulation, at early time points and in clinically scrapie-affected animals, despite all other cell types within the lymphoid tissue retaining expression of PrP^C and therefore potentially capable of replicating the scrapie agent. It can therefore be concluded that FDCs do not acquire PrP^d on their surface after replication on another cell type.

Animals with PrP^C expression switched on exclusively in FDCs were also inoculated with the ME7 scrapie agent to determine if PrP^C expression exclusively on FDCs was sufficient to support scrapie replication in the lymphoid tissue and therefore determine whether FDCs actively replicate the TSE agent (Chapter 7). Animals with PrP^C expression exclusively on FDCs showed deposition of PrP^d on the FDC network which increased over time at a comparable level to that observed in $PrP^{+/-}$ hemizygous controls. Whereas control animals with PrP^C expression switched on only in B lymphocytes had little/no detectable PrP^d labelling and again any labelling present was only found within the TBMs. These data show that PrP^C expression exclusively on FDCs is sufficient to support TSE agent replication in the lymphoid tissue.

Taken together, data from these studies shows that FDCs do not acquire PrP^d from other cell types within the lymphoid tissue and instead actively replicate the TSE agent. FDCs appear to be responsible for replicating the TSE agent to sufficient levels that allow neuroinvasion and CNS disease. However are the FDCs themselves actually infected with the scrapie agent? Data from this thesis has shown that FDCs do not accumulate PrP^d from other cells in the lymphoid tissues and instead actively replicate the scrapie agent themselves. However, ultrastructural analysis shows PrP^d accumulations on the FDCs have only ever been observed on the plasmalemma, not within the cytoplasm (Jeffrey, McGovern et al. 2000; McGovern, Mabbott et al. 2009). Furthermore, there is a general belief that although PrP^d accumulation on neurones causes cell death by an unknown mechanism, PrP^d accumulation on the FDCs does not have an obvious toxic effect. As the scrapie agent is not replicating within the cell itself and replication does not appear to have a pathological effect on

the FDCs, one could argue that FDCs merely provide a platform for replication prior to neuroinvasion and infection of cells within the CNS.

Recent studies investigating the effects of scrapie infection on cells of the immune system have shown that replication of the TSE agent does effect the morphology of the FDCs and therefore may have a subtle effect on immune function that as of yet has not been investigated closely. Studies looking at PrP^d accumulation on the FDCs of sheep noted abnormal convolutions in the plasmalemma and abnormal clustering of immune complexes which co-localised with areas of PrP^d accumulation on the FDC surface. An increased number of exosomes was also observed around FDCs showing morphological changes due to PrP^d accumulation, some of which contained PrP^d (McGovern and Jeffrey 2007). Morphological changes to the FDCs leading to the shedding of the PrP^d in exosomes may be one possible mechanism of neuroinvasion of the scrapie agent. These observations were confirmed in the FDCs of scrapie-affected mice, however the increased number of exosomes in the follicle was not observed in these models (Jeffrey, McGovern et al. 2000; McGovern, Brown et al. 2004). More recent studies showed that accumulation of PrP^d in scrapie-affected mice led to disruptions in the FDC maturation cycle, abnormal ubiquitin in cell membranes and an excessive accumulation of Igs which could therefore impact on immune function (McGovern, Mabbott et al. 2009a). The transfer of PrP^d to mature Ab-producing B lymphocytes has also been noted in the follicles of scrapie-affected sheep (McGovern and Jeffrey 2007). Additional studies have reported changes in B lymphocyte subsets in response to scrapie infection in sheep, which again could impact on immune function (Eaton, Rocchi et al. 2007; Eaton, Anderson et al. 2009). Although PrP^d does not appear to be internalised within the FDCs, FDCs actively

replicate the TSE agent and show pathological changes in response to accumulation of the agent on their surface, which in turn may impact on immune function. For this reason, FDCs could be considered to be infected with the TSE agent as they are adversely affected as a consequence.

8.6 FDC-independent TSE pathogenesis

Although data from this thesis and many previous studies show that FDCs have an important role in replicating the TSE agent in the lymphoid tissue, in some cases TSE infections appear to be able to bypass the need for replication upon FDCs. Some naturally acquired infections, for example BSE in cattle and atypical BSE and scrapie, develop clinical disease in the CNS without any apparent replication in the lymphoid tissue (Somerville, Birkett et al. 1997; Buschmann, Biacabe et al. 2004; Benestad, Arsac et al. 2008). Recent experimental studies created granulomas in the skin of mice prior to inoculation with scrapie (Heikenwalder, Kurrer et al. 2008). Accumulation of PrP^{Sc} and infectivity occurred within the granulomas in association with stromal cells. These stromal cells did not express the characteristic FDC markers such as C1q, CD35, FDC-M1, FDC-M2 or mRNA for *Mfge8* and therefore this study was used as an example of FDC-independent replication of the scrapie agent. However, stromal cells within the granuloma were dependent on LT signalling, expressed high levels of PrP^{C} and organised B lymphocytes within the granuloma into follicle type structures. Therefore although the commonly used FDC markers were not present, these cells demonstrated many of the functional abilities FDCs have within the lymphoid tissue. If these cells were characterised by function they would possibly be described as FDCs. The lack of FDC markers in an artificially induced

tertiary lymphoid tissue is possibly not a great example of non-FDC associated replication of the scrapie agent as natural disease-induced chronic inflammation with formation of ectopic lymphoid follicles have shown development of PrP^{C} expressing FDCs which are capable of replicating PrP^{Sc} agent upon co-infection with the scrapie agent (Heikenwalder, Zeller et al. 2005; Ligios, Sigurdson et al. 2005).

Evidence from other studies has implied that there may be variations between TSE agent strains regarding whether replication on the FDCs is required prior to neuroinvasion. Studies described previously in this chapter (and thesis) demonstrated that expression of PrP^{C} by the FDCs is essential for successful pathogenesis of the ME7 scrapie agent in mice, whereas PrP^{C} expression on lymphocytes was irrelevant (Brown, Stewart et al. 1999). However, similar studies using the RML scrapie isolate inoculated into *Prnp*$^{-/-}$ mice reconstituted with *Prnp*$^{+/+}$ haematopoietic stem cells showed successful neuroinvasion of the scrapie agent despite the FDCs lacking PrP^{C} expression in this model (Blattler, Brandner et al. 1997). This study may demonstrate that different strains of TSE agent have different requirements for replication in the periphery prior to neuroinvasion. However, an important factor to consider is that this study used a high TSE agent dose for inoculation, 100 µl of a 1% brain homogenate (Blattler, Brandner et al. 1997) in comparison to 20 µl of a 1% brain homogenate used in the ME7 study (Brown, Stewart et al. 1999). Previous experiment using SCID mice have shown that the need for TSE agent replication in the periphery can be overcome by inoculating with a high dose of the TSE agent (Fraser, Brown et al. 1996). Therefore it may be that levels of infectivity in the innocula used in the RML experiments were sufficient to allow neuroinvasion without further replication in the periphery.

With the exception of some natural disease models, such as BSE in cattle, the majority of experimental evidence suggests that FDCs provide a crucial site for peripheral replication of the TSE agent until sufficient levels of infectivity are reached that allow neuroinvasion. Data in this thesis shows, the ME7 scrapie agent, cannot replicate in lymphoid tissues containing PrP^C deficient FDC despite all other cell types retaining PrP^C expression. However, other TSE strains have not been tested in this thesis, so the prospect of strain differences cannot be completely discounted.

8.7 Localisation of the TSE agent to the follicle

Another uncertainty in TSE disease pathogenesis is how the TSE agent localises to the FDC network prior to replication and subsequent neuroinvasion. Data from this thesis has shown that the scrapie agent can localise to PrP^C deficient FDCs in the follicles at the same time points as that seen in PrP^C expressing FDCs (Chapter 5). This demonstrates that PrP^C expression by FDCs is not required for the scrapie agent to initially localise to the FDC network. Furthermore, when PrP^C was expressed exclusively on the FDC network, the scrapie agent could again localise to the follicle at the same time points as *Prnp*$^{+/-}$ controls (Chapter 7). This shows that PrP^C expression by other cell types is not required for the transport of the agent to the FDC network. These data suggest that PrP^C is neither required as a ligand for the transportation of the scrapie agent to the follicle nor for initial uptake by the FDCs. This is in agreement with previous studies which showed that localisation of the scrapie agent to the follicle was dependent on CD21/35 expression on the stromal compartment of the spleen, whereas expression status of PrP^C was irrelevant (Zabel, Heikenwalder et al. 2007).

Some experimental evidence suggests that the TSE agent may use cell-mediated transport to localise to the follicle. Macrophages have been shown to have the ability to take up the PrP^d and after peripheral inoculation with scrapie are also capable of harbouring infectivity (Jeffrey, McGovern et al. 2000; Manuelidis, Zaitsev et al. 2000). However, most studies agree that rather than transporting the agent, PrP^d is taken up into lysosomes and partially degraded (Jeffrey, Martin et al. 2003; McGovern, Mabbott et al. 2009a). Removal of macrophages leads to increased replication of the TSE agent suggesting they are involved in clearing the agent (Beringue, Demoy et al. 2000; Maignien, Shakweh et al. 2005). DCs are another possible candidate for transport of the TSE agent to the lymphoid tissues. Previous studies have indicated that after oral exposure DCs take up the scrapie agent and transport it to the draining lymphoid tissues (Huang, Farquhar et al. 2002; Huang and MacPherson 2004; Mohan, Hopkins et al. 2005; Raymond, Aucouturier et al. 2007). Recent studies have shown that uptake of the scrapie agent by DCs is facilitated by complement components (Flores-Langarica, Sebti et al. 2009). However, even after the temporary depletion of DCs at the time of exposure, some animals eventually succumbed to disease, therefore it is possible that cell-free transport of the TSE agent to the follicle is also possible (Raymond, Aucouturier et al. 2007).

After peripheral inoculation, the TSE agent may be transported via the blood directly to the spleen, by cell-free mechanisms. Studies investigating which size of PrP^d aggregates are most infectious discovered that particles of 300-600 KDa in size contained the most infectivity. Therefore it is likely that infectivity and the TSE agent itself is associated with PrP^d fragment of this size. The pathway of protein/antigen processing by the spleen varies in accordance with the size of the antigen. Small

soluble protein antigens, less than 70 KDa, are transported through the spleen via the conduits to the follicle (Pape, Catron et al. 2007; Roozendaal, Mempel et al. 2009). Whereas large antigen, greater than 70KDa, is trapped by sub-capsular sinus (SCS) macrophages in the lymph nodes or marginal zone (MZ) macrophages in the spleen. Antigen is then removed from the SCS/ MZ macrophages by follicular B lymphocytes which then traffic to the follicle where antigen is taken up by FDCs (Carrasco and Batista 2007; Phan, Grigorova et al. 2007; Phan, Green et al. 2009; Roozendaal, Mempel et al. 2009). Therefore, it is likely that cell free infective scrapie agent is taken up by SCS macrophages and trafficked via B lymphocytes to the FDCs. This mechanism of antigen transport has been shown to be dependent on complement receptors (Phan, Grigorova et al. 2007). In agreement with the scrapie agent using this mechanism of transport to locate to the follicle, previous studies have shown that complement components aid scrapie agent localisation to the follicle and that depletion of these factors or their receptors can delay neuroinvasion and clinical disease (Klein, Kaeser et al. 2001; Mabbott, Bruce et al. 2001).

In all of these studies examining the trafficking of the TSE agent to the FDCs, impairment in various mechanisms causes a delay in neuroinvasion but not a prevention of disease. It appears that the TSE agent can use various mechanisms to get to the follicle and replicate on the FDCs and no one mechanism is solely responsible for disease pathogenesis to occur.

8.8 The role of B lymphocytes in TSE pathogenesis

Experimental evidence from previous studies indicated that B lymphocytes may have a role in replicating the scrapie agent in the lymphoid tissue prior to neuroinvasion (Klein, Frigg et al. 1997). However, it was subsequently determined that effects of B cell deficiency on pathogenesis in this model was due to the indirect effect of having a lack of B lymphocyte-derived LTs to maintain mature FDC networks. Subsequent studies have shown that PrP^C expressing B lymphocytes are not essential for effective pathogenesis of the scrapie agent (Brown, Stewart et al. 1999; Montrasio, Cozzio et al. 2001). This is in agreement with data from this thesis which found that switching off PrP^C exclusively on $CD21^+$ B lymphocytes had no effect on PrP^d deposition in the spleen after inoculation with the ME7 scrapie agent (Chapter 5). Furthermore, PrP^C expression exclusively by $CD21^+$ B lymphocytes did not allow replication of the scrapie agent in the spleen (Chapter 7). Data from previous studies and this thesis imply that B lymphocytes do not have a role in replicating the TSE agent in peripheral lymphoid tissue. However, this does not mean that they have no involvement in TSE disease. As discussed in Section 8.7, B lymphocytes may be involved in the initial transportation of the TSE agent from SCS macrophages to the FDC networks via complement-dependent mechanisms (Carrasco and Batista 2007; Phan, Grigorova et al. 2007; Phan, Green et al. 2009; Roozendaal, Mempel et al. 2009). Furthermore, experiments have shown that PrP^d is transferred from FDCs to the surface membranes of B lymphocytes in the germinal centres of scrapie-affected mice (McGovern and Jeffrey 2007), In addition PrP^d was found associated with $CD21^+$ B lymphocytes in the blood of scrapie-affected sheep. Therefore it is possible that B lymphocytes are involved in trafficking the scrapie agent before or after replication on the lymphoid

tissues. One experiment that could have been carried out in the studies in this thesis would be to isolate $CD21^+$ B lymphocytes from the blood and spleen of scrapie affected animals and measure the levels of PrP labelling by FACS analysis. This could provide data on whether switching PrP^C expression on or off specifically on $CD21^+$ B lymphocytes, or on $CD21^+$ B lymphocytes and FDCs, had any effect on the accumulation of PrP^d on the $CD21^+$ B lymphocytes. It could be used as one possible method to determine if $CD21^+$ B lymphocytes were responsible for trafficking the agent after replication on the FDC networks. Unfortunately there was not sufficient time to carry out these experiments in this thesis, but it may provide a method for addressing this question in future studies.

8.9 The role of tingible body macrophages in TSE pathogenesis

TBMs are large phagocytic cells found in the germinal centres of secondary lymphoid tissues which contain phagocytosed, apoptotic cells known as tingible bodies (Swartzendruber and Congdon 1963). Labelling of PrP^d has been found within TBMs after inoculation with scrapie in mice (Jeffrey, McGovern et al. 2000) and sheep (Jeffrey, Martin et al. 2001) and also in lymphoid tissues taken from individuals who have died from vCJD infection (Hilton, Ghani et al. 2004). In this thesis, PrP^d was found in association with TBMs when PrP^C was switched off on the FDCs after ip inoculation with the ME7 scrapie agent (Chapter 5). In addition, when PrP^C was switched on exclusively on $CD21^+$ B lymphocytes, PrP^d was only found in association with TBMs in the lymphoid tissue (Chapter 7). These data suggest that in the absence of TSE agent replication by the FDC network, TBMs scavenge the PrP^d that has located to the FDC network probably via complement dependent mechanisms (See

Section 8.6). Indeed, previous studies have shown that TBMs are capable of taking up portions of immune complex coated membrane from the FDCs (Tew, Kosco et al. 1989; Szakal and Tew 1992). In this thesis, it is not certain whether the PrP^{d} labelling found within the TBMs will act as a reservoir of infection, harbouring the scrapie agent and allowing a delayed neuroinvasion, or whether PrP^{d} within the TBMs is degraded and infectivity is destroyed. Cre^{+ve} →*CD21-cre*$PrP^{fl/-}$ mice, which have PrP^{C} switched off exclusively in FDCs, have been ip inoculated with the ME7 scrapie agent and left until clinical disease develops or animals need to be culled due to old age. However, at the time of writing, these animals have not succumbed to clinical disease or age, so as of yet no conclusions can be drawn from these experiments. However, previous studies have shown that PrP^{d} found within the TBMs is truncated with a loss of 23-90 aa from the N-terminal region (Jeffrey, Martin et al. 2003; McGovern, Mabbott et al. 2009a). This suggests that the TBMs are degrading the scrapie agent after uptake from the FDCs. In scrapie infections, an initial exponential growth of PrP^{d} deposition and infectivity occurs initially in the spleen which is then followed by a plateau. Many suggestions have been made as to why this effect is seen. If TBMs in the follicle are responsible for degrading the scrapie agent, it may be that a balance is eventually reached between replication of the scrapie agent on the FDCs and degradation by the TBMs.

8.10 Conclusions

In conclusion, data from this thesis have confirmed that FDCs play a crucial role in replicating the scrapie agent prior to neuroinvasion. The *CD21-cre*$PrP^{fl/-}$ transgenic mouse line enabled PrP^{C} expression to be switched off exclusively on the FDC

network after irradiation and transfer of non-Cre-expressing bone marrow. This model was used to determine whether FDCs simply acquire the TSE agent on their surface after accumulation on another cell type. Characterisation of this model showed efficient removal of PrP^{C} specifically on the FDCs with no additional effects on FDC status or function, or on the general microarchitecture of the lymphoid tissue. These animals were infected with the ME7 scrapie agent via ip inoculation. In these animals, PrP^{d} initially located to the FDC networks, however the TSE agent was unable to replicate on the PrP^{C} deficient FDCs and the agent was taken up by neighbouring TBMs and possibly degraded (Chapter 5). These animals were also infected directly into the CNS via ic injection and developed clinical TSE disease with positive neuropathology in the brain. This demonstrated that the transgenic animals were equally susceptible to TSE disease as their WT counterparts and that any effect on disease pathogenesis in the lymphoid tissue was due to PrP^{C} deficient FDCs and not a general resistance to TSE disease. As PrP^{C} deficient FDCs did not show deposition of the TSE agent on their surface which increased as disease progressed, it was concluded that FDCs do not acquire the TSE agent from other stromal, neural or lymphoid cells within the lymphoid tissue which retained PrP^{C} expression in the *CD21-cre*$PrP^{fl/-}$ transgenic mouse model. PrP^{C}-expressing FDCs are essential for replicating the scrapie agent in the lymphoid tissue.

Following on from these studies, it was determined whether PrP^{C} expression exclusively on FDCs was sufficient to support replication of the scrapie agent in the lymphoid tissue. The *CD21-cre*$PrP^{stop/-}$ transgenic mouse line enabled PrP^{C} to be expressed exclusively on the FDC network after irradiation and transfer of non-Cre-expressing bone marrow. Characterisation of this model demonstrated that PrP^{C} was

efficiently and specifically expressed on the FDC networks and again no additional effects were observed on FDC status or general microarchitecture of the lymphoid tissue. After infection of these animals with the ME7 scrapie agent via ip inoculation, accumulation of PrP^{d} occurred on FDCs when PrP^{C} was restricted to the FDC network. Furthermore, this accumulation increased over time suggesting active replication of the scrapie agent by the FDCs. Taken together, data from both of these transgenic mouse models shows that FDCs do not acquire PrP^{d} and infectivity from other cells within the lymphoid tissue. Furthermore, PrP^{C} expression exclusively on FDCs is sufficient to support replication of the scrapie agent in the lymphoid tissue. From these data we can conclude that PrP^{C} expressing FDCs actively replicate the TSE agent in the lymphoid tissue after peripheral exposure to scrapie.

Bibliography

Ahmed, R. and D. Gray (1996). "Immunological Memory and Protective Immunity: Understanding Their Relation." Science **272**(5258): 54-60.

Alper, T., D. A. Haig, et al. (1966). "The exceptionally small size of the scrapie agent." Biochemical and Biophysical Research Communications **22**(3): 278-284.

Anderson, R. M., C. A. Donnelly, et al. (1996). "Transmission dynamics and epidemiology of BSE in British cattle." Nature **382**(6594): 779-788.

Andrasfalvy, M., J. Prechl, et al. (2002). "Mucosal type mast cells express complement receptor type 2 (CD21)." Immunology Letters **82**(1-2): 29-34.

Andreoletti, O., P. Berthon, et al. (2000). "Early accumulation of PrPSc in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie." J Gen Virol **81**(12): 3115-3126.

Aucouturier, P., F. Geissmann, et al. (2001). "Infected splenic dendritic cells are sufficient for prion transmission to the CNS in mouse scrapie." J. Clin. Invest. **108**(5): 703-708.

Aude-Garcia, C., C. L. Villiers, et al. (2011). "Enhanced susceptibility of T lymphocytes to oxidative stress in the absence of the cellular prion protein." Cellular and Molecular Life Sciences **68**(4): 687-696.

Ballerini, C., P. Gourdain, et al. (2006). "Functional Implication of Cellular Prion Protein in Antigen-Driven Interactions between T Cells and Dendritic Cells." The Journal of Immunology **176**(12): 7254-7262.

Balogh, P. r., Y. Aydar, et al. (2001). "Ontogeny of the Follicular Dendritic Cell Phenotype and Function in the Postnatal Murine Spleen." Cellular Immunology **214**(1): 45-53.

Barclay, G. R., J. Hope, et al. (1999). "Distribution of cell-associated prion protein in normal adult blood determined by flow cytometry." British Journal of Haematology **107**(4): 804-814.

Baron, T., A. G. Biacabe, et al. (2007). "Atypical transmissible spongiform encephalopathies (TSEs) in ruminants." Vaccine **25**(30): 5625-30.

Barria, M. A., A. Mukherjee, et al. (2009). "*De novo* generation of infectious prions *in vitro* produces a new disease phenotype." PLoS pathogens **5**(5): 2-10.

Barron, R. M., S. L. Campbell, et al. (2007). "High Titers of Transmissible Spongiform Encephalopathy Infectivity Associated with Extremely Low Levels of PrP^{Sc} in Vivo." J. Biol. Chem. **282**(49): 35878-35886.

Barron, R. M. and J. C. Manson (2003). "A gene-targeted mouse model of P102L Gerstmann-Sträusser-Scheinker syndrome." Clinics in Laboratory Medicine **23**: 161-173.

Benestad, S. L., J.-N. Arsac, et al. (2008). "Atypical/ Nor98 scrapie: properties of the agent, genetics and epidemiology." Veterinary Research **39**(19).

Blattler, T., S. Brandner, et al. (1997). "PrP-expressing tissue required for transfer of scrapie infectivity from spleen to brain." Nature **389**(6646): 69-73.

Block, M. L., L. Zecca, et al. (2007). "Microglia-mediated neurotoxicity: uncovering the molecular mechanisms." Nat Rev Neurosci **8**(1): 57-69.

Bolton, D., McKinley, MP and Prusiner, SB (1982). "Identification of a protein that purifies with the scrapie prion." Science **218**(4579): 1309-1311.

Bolton, D. C., M. P. McKinley, et al. (1982). "Identification of a protein that purifies with the scrapie prion." Science **218**(4579): 1309-1311.
Bolton, D. C., R. K. Meyer, et al. (1985). "Scrapie PrP 27-30 is a sialoglycoprotein." J. Virol. **53**(2): 596-606.
Bounhar, Y., Y. Zhang, et al. (2001). "Prion Protein Protects Human Neurons against Bax-mediated Apoptosis." Journal of Biological Chemistry **276**(42): 39145-39149.
Bradford, B. M., N. L. Tuzi, et al. (2009). "Dramatic Reduction of PrPC Level and Glycosylation in Peripheral Nerves following PrP Knock-Out from Schwann Cells Does Not Prevent Transmissible Spongiform Encephalopathy Neuroinvasion." The Journal of Neuroscience **29**(49): 15445-15454.
Brotherston, J. G., C. C. Renwick, et al. (1968). "Spread of scrapie by contact to goats and sheep." Journal of Comparative Pathology **78**(1): 9-17.
Brown, K. L., K. Stewart, et al. (1999). "Scrapie replication in lymphoid tissues depends on prion protein-expressing follicular dendritic cells." Nature Medicine **5**(11): 1308-1312.
Brown, K. L., G. J. Wathne, et al. (2009). "The Effects of Host Age on Follicular Dendritic Cell Status Dramatically Impair Scrapie Agent Neuroinvasion in Aged Mice." J Immunol: jimmunol.0802695.
Brown, P. and R. Bradley (1998). "1755 and all that: a historical primer of transmissible spongiform encephalopathy." BMJ **317**(7174): 1688-1692.
Brown, P., R. G. Rohwer, et al. (1986). "Newer Data on the Inactivation of Scrapie Virus or Creutzfeldt-Jakob Disease Virus in Brain Tissue." Journal of Infectious Diseases **153**(6): 1145-1148.
Bruce, M. E. (1993). "Scrapie strain variation and mutation." British Medical Bulletin **49**(4): 822-838.
Bruce, M. E., A. Boyle, et al. (2002). "Strain characterization of natural sheep scrapie and comparison with BSE." J Gen Virol **83**(3): 695-704.
Bruce, M. E., K. L. Brown, et al. (2000). "Follicular dendritic cells in TSE pathogenesis." Immunology Today **21**(9): 442-446.
Bruce, M. E. and H. Fraser (1991). "Scrapie strain variation and its implications." Current Topics in Microbiology and Immunology **172**: 125-38.
Bruce, M. E., P. A. McBride, et al. (1989). "Precise targeting of the pathology of the sialoglycoprotein, PrP, and vacuolar degeneration in mouse scrapie." Neuroscience Letters **102**(1): 1-6.
Bruce, M. E., I. McConnell, et al. (1991). "The disease characteristics of different strains of scrapie in Sinc congenic mouse lines: implications for the nature of the agent and host control of pathogenesis." J Gen Virol **72**(3): 595-603.
Bruce, M. E., I. McConnell, et al. (2001). "Detection of variant Creutzfeldt-Jakob disease infectivity in extraneural tissues." The Lancet **358**(9277): 208-209.
Bruce, M. E., R. G. Will, et al. (1997). "Transmissions to mice indicate that /`new variant/' CJD is caused by the BSE agent." Nature **389**(6650): 498-501.
Büeler, H., A. Aguzzi, et al. (1993). "Mice devoid of PrP are resistant to scrapie." Cell **73**: 1339-1347.
Bueler, H., M. Fischer, et al. (1992). "Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein." Nature **356**(6370): 577-582.
Burthem, J., B. Urban, et al. (2001). "The normal cellular prion protein is strongly expressed by myeloid dendritic cells." Blood **98**(13): 3733-3738.

Buschmann, A., A. G. Biacabe, et al. (2004). "Atypical scrapie cases in Germany and France are identified by discrepant reaction patterns in BSE rapid tests." Journal of Virological Methods **117**(1): 27-36.

Campana, V., D. Sarnataro, et al. (2005). "The highways and byways of prion protein trafficking." Trends in Cell Biology **15**(2): 102-111.

Carp, R. I. and S. M. Callaghan (1981). "In vitro interaction of scrapie agent and mouse peritoneal macrophages." Intervirology **16**(1): 8-13.

Carp, R. I. and S. M. Callaghan (1982). "Effect of mouse peritoneal macrophages on scrapie infectivity during extended in vitro incubation." Intervirology **17**: 201-207.

Cashman, N. R., R. Loertscher, et al. (1990). "Cellular isoform of the scrapie agent protein participates in lymphocyte activation." Cell **61**(1): 185-192.

Caughey, B., Race, RE and Chesebro, B (1988). "Detection of prion protein mRNA in normal and scrapie-infected tissues and cell lines." Journal of General Virology **69**: 711-716.

Caughey, B. W., A. Dong, et al. (1991). "Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy." Biochemistry **30**(31): 7672-7680.

Cerpa, W., L. Varela-Nallar, et al. (2005). "Is there a role for copper in neurodegenerative diseases?" Molecular Aspects of Medicine **26**(4-5): 405-420.

Chaplin, D. D. and Y.-x. Fu (1998). "Cytokine regulation of secondary lymphoid organ development." Current Opinion in Immunology **10**(3): 289-297.

Chesebro, B., R. Race, et al. (1985). "Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain." Nature **315**(6017): 331-333.

Chiarini, L. B., A. R. O. Freitas, et al. (2002). "Cellular prion protein transduces neuroprotective signals." EMBO J **21**(13): 3317-3326.

Clarke, M. C. and D. A. Haig (1966). "Attempts to demonstrate neutralising antibodies in the sera of scrapie-affectedsheep." Veterinary Record **78**: 647-49.

Clarke, M. C. and D. A. Haig (1971). "Multiplication of scrapie agent in mouse spleen." Research in Veterinary Science **12**: 195-197.

Clarke, M. C. and R. H. Kimberlin (1984). "Pathogenesis of mouse scrapie: Distribution of agent in the pulp and stroma of infected spleens." Veterinary Microbiology **9**(3): 215-225.

Coitinho, A. S., R. Roesler, et al. (2003). "Cellular prion protein ablation impairs behavior as a function of age." NeuroReport **14**(10): 1375-1379 10.1097/01.wnr.0000078541.07662.90.

Colling, S. B., J. Collinge, et al. (1996). "Hippocampal slices from prion protein null mice: disrupted Ca2+-activated K+ currents." Neuroscience Letters **209**(1): 49-52.

Collinge, J., K. C. L. Sidle, et al. (1996). "Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD." Nature **383**(6602): 685-690.

Collinge, J., M. A. Whittington, et al. (1994). "Prion protein is necessary for normal synaptic function." Nature **370**(6487): 295-297.

Comber, T. (1772). Real Improvements in Agriculture. London, W. Nicoll.

Creutzfeldt, H. G. (1920). "Uber eine eigenartige herdformige Erkrankung des Zentralnervensystems. Vorlaufige Mitteilung." Zeitschrift fur die gesamte Neurologie und Psychiatrie **57**: 1-18.

Cuillé, J. and P.-L. Chelle (1936). "Pathologie animale- La maladie dite tremblante du mouton *est-elle inoculable?* ." Comptes rendus hebdomadaires des séances de l'Académie des Sciences **203**(2).
Cuillé, J. and P.-L. Chelle (1938). "La tremblante du mouton est-elle déterminée par un virus filtrable?" Comptes rendus hebdomadaires des séances de l'Académie des Sciences **206**(2): 1687-1688.
Cuillé, J. and P.-L. Chelle (1939). "Transmission expérimentale de la tremblante à la chévre." Comptes rendus hebdomadaires des séances de l'Académie des Sciences **208**(1): 1058-1060.
Denzer, K., M. van Eijk, et al. (2000). "Follicular Dendritic Cells Carry MHC Class II-Expressing Microvesicles at Their Surface." J Immunol **165**(3): 1259-1265.
Dickinson, A. G., H. Fraser, et al. (1975). "Scrapie incubation time can exceed natural lifespan." Nature **256**(5520): 732-733.
Dickinson, A. G. and J. M. K. Mackay (1964). "Genetical control of the incubation period in mice of the neurological disease, scrapie." Heredity **19**(2): 279-288.
Dickinson, A. G. and V. M. H. Meikle (1971). "Host genotype and agent effects in scrapie incubations: change in allelic interactions with different strains of agent." Molecular and General Genetics **112**: 73-79.
Dickinson, A. G., V. M. H. Meikle, et al. (1968). "Identification of a gene which controls the incubation period of some strains of scrapie agent in mice." Journal of Comparative Pathology **78**(3): 293-299.
Dickinson, A. G. and G. W. Outram (1988). "Genetic aspects of unconventional virus infections: the basis of the virino hypothesis." Ciba Foundation symposium **135**: 63-83.
Dickinson, A. G., J. T. Stamp, et al. (1968). "Some factors controlling the incidence of scrapie in Cheviot sheep injected with a Cheviot-passaged scrapie agent." Journal of Comparative Pathology **78**(3): 313-321.
Diringer, H., H. Gelderblom, et al. (1983). "Scrapie infectivity, fibrils and low molecular weight protein." Nature **306**(5942): 476-478.
Dlakic, W. M., E. Grigg, et al. (2007). "Prion Infection of Muscle Cells In Vitro." J. Virol. **81**(9): 4615-4624.
Dürig, J., A. Giese, et al. (2000). "Differential constitutive and activation-dependent expression of prion protein in human peripheral blood leucocytes." British Journal of Haematology **108**(3): 488-495.
Eklund, C. M., R. C. Kennedy, et al. (1967). "Pathogenesis of scrapie virus infection in the mouse." J. Infect. Dis. **117**: 15-22.
Ermonval, M., S. Mouillet-Richard, et al. (2003). "Evolving views in prion glycosylation: functional and pathological implications." Biochimie **85**(1-2): 33-45.
Fang, Y., C. Xu, et al. (1998). "Expression of Complement Receptors 1 and 2 on Follicular Dendritic Cells Is Necessary for the Generation of a Strong Antigen-Specific IgG Response." The Journal of Immunology **160**(11): 5273-5279.
Farquhar, C. F., R. A. Somerville, et al. (1998). "Straining the prion hypothesis." Nature **391**(6665): 345-346.
Farquhar, C. F., R. A. Somerville, et al. (1989). "Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy." Journal of Virological Methods **24**(1-2): 215-221.

Fischer, M., T. Rulicke, et al. (1996). "Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie." The EMBO journal **15**(6): 1255-64.
Flores-Langarica, A., Y. Sebti, et al. (2009). "Scrapie Pathogenesis: The Role of Complement C1q in Scrapie Agent Uptake by Conventional Dendritic Cells." J Immunol **182**(3): 1305-1313.
Foster, J., C. McKenzie, et al. (2006). "Lateral transmission of natural scrapie to scrapie-free New Zealand sheep placed in an endemically infected UK flock." Vet Rec. **159**(19): 633-634.
Foster, J. D., N. Hunter, et al. (1996). "Observations on the transmission of scrapie in experiments using embryo transfer." Veterinary Record **138**(23): 559-562.
Fox, K. A., J. E. Jewell, et al. (2006). "Patterns of PrPCWD accumulation during the course of chronic wasting disease infection in orally inoculated mule deer (Odocoileus hemionus)." J Gen Virol **87**(11): 3451-3461.
Fraser, H., K. L. Brown, et al. (1996). "Replication of scrapie in spleens of SCID mice follows reconstitution with wild-type mouse bone marrow." J Gen Virol **77**(8): 1935-1940.
Fraser, H. and A. G. Dickinson (1970). "Pathogenesis of Scrapie in the Mouse: the Role of the Spleen." Nature **226**(5244): 462-463.
Fraser, H. and A. G. Dickinson (1973). "Scrapie in mice : Agent-strain differences in the distribution and intensity of grey matter vacuolation." Journal of Comparative Pathology **83**(1): 29-40.
Fraser, H. and C. F. Farquhar (1987). "Ionising radiation has no influence on scrapie incubation period in mice." Veterinary Microbiology **13**(3): 211-223.
Fraser, H. a. D., AG (1978). "Studies of the lymphoreticular system in the pathogenesis of scrape: The role of the spleen and the thymus." Journal of Comparitive Pathology **88**: 563-573.
Fraser, H. a. F., CF (1987). "Ionising radiation has no influence on scrapie incubation period in mice." Veterinary Microbiology **13**(3): 277-223.
Gerstmann, J., E. Straussler, et al. (1936). "Uber eine Eigenartige hereditaer-familiaere Erkrankung des Zentralnervensystem." Zeitschrift fur die gesamte Neurologie und Psychiatrie **154**: 736-62.
Glatzel, M., E. Abela, et al. (2003). "Extraneural Pathologic Prion Protein in Sporadic Creutzfeldt-Jakob Disease." N Engl J Med **349**(19): 1812-1820.
Glatzel, M., F. L. Heppner, et al. (2001). "Sympathetic Innervation of Lymphoreticular Organs Is Rate Limiting for Prion Neuroinvasion." Neuron **31**(1): 25-34.
Glaysher, B. R. and N. A. Mabbott (2007). "Role of the GALT in Scrapie Agent Neuroinvasion from the Intestine." J Immunol **178**(6): 3757-3766.
Goldfarb, L. G., R. B. Petersen, et al. (1992). "Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism." Science **258**(5083): 806-808.
Goldrath, A. W., C. J. Luckey, et al. (2004). "The molecular program induced in T cells undergoing homeostatic proliferation." Proceedings of the National Academy of Sciences of the United States of America **101**(48): 16885-16890.
Gordon, W. S. (1946). "Louping-ill, tick-borne fever and scrapie." Veterinary Record **58**(47): 516-525.
Gray, D., M. H. Kosco, et al. (1991). "Novel pathways of antigen presentation for the maintenance of memory." Int. Immunol. **3**(2): 141-148.

Gray, D. and P. Matzinger (1991). "T cell memory is short-lived in the absence of antigen." The Journal of Experimental Medicine **174**(5): 969-974.
Gray, D. and H. Skarvall (1988). "B-cell memory is short-lived in the absence of antigen." Nature **336**(6194): 70-73.
Haddon, D. J., M. R. Hughes, et al. (2009). "Prion Protein Expression and Release by Mast Cells After Activation." Journal of Infectious Diseases **200**(5): 827-831.
Hanayama, R., M. Tanaka, et al. (2004). "Autoimmune Disease and Impaired Uptake of Apoptotic Cells in MFG-E8-Deficient Mice." Science **304**(5674): 1147-1150.
Head, M. W., V. Northcott, et al. (2003). "Prion Protein Accumulation in Eyes of Patients with Sporadic and Variant Creutzfeldt-Jakob Disease." Investigative Ophthalmology & Visual Science **44**(1): 342-346.
Head, M. W., D. L. Ritchie, et al. (2004). "Peripheral Tissue Involvement in Sporadic, Iatrogenic, and Variant Creutzfeldt-Jakob Disease: An Immunohistochemical, Quantitative, and Biochemical Study." The American journal of pathology **164**(1): 143-153.
Hegde, R. S., J. A. Mastrianni, et al. (1998a). "A Transmembrane Form of the Prion Protein in Neurodegenerative Disease." Science **279**(5352): 827-834.
Hegde, R. S., S. Voigt, et al. (1998b). "Regulation of Protein Topology by trans-Acting Factors at the Endoplasmic Reticulum." Molecular cell **2**(1): 85-91.
Heggebo, R., L. Gonzalez, et al. (2003). "Disease-associated PrP in the enteric nervous system of scrapie-affected Suffolk sheep." J Gen Virol **84**(5): 1327-1338.
Heikenwalder, M., M. O. Kurrer, et al. (2008). "Lymphotoxin-dependent prion replication in inflammatory stromal cells of granulomas." Immunity **29**: 998-1008.
Heikenwalder, M., N. Zeller, et al. (2005). "Chronic Lymphocytic Inflammation Specifies the Organ Tropism of Prions." Science **307**(5712): 1107-1110.
Heinen, E., A. Bosseloir, et al. (1995). "Follicular dendritic cells: Origin and function." Current Topics in Microbiology and Immunology **201**: 15-47.
Hill, A. F., R. J. Butterworth, et al. (1999). "Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples." The Lancet **353**(9148): 183-189.
Hill, A. F., M. Zeidler, et al. (1997). "Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy." Lancet **349**: 99-100.
Hilton, D. A., E. Fathers, et al. (1998). "Prion immunoreactivity in appendix before clinical onset of variant Creutzfeldt-Jakob disease." The Lancet **352**(9129): 703-704.
Hilton, D. A., A. C. Ghani, et al. (2004). "Prevalence of lymphoreticular prion protein accumulation in UK tissue samples." The Journal of Pathology **203**(3): 733-739.
Holada, K. and J. G. Vostal (2000). "Different levels of prion protein (PrPc) expression on hamster, mouse and human blood cells." British Journal of Haematology **110**(2): 472-480.
Hope, J., L. J. D. Morton, et al. (1986). "The major protein of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP)." EMBO Journal **5**: 2591-2597.
Hornshaw, M. P., J. R. McDermott, et al. (1995). "Copper Binding to the N-Terminal Tandem Repeat Region of Mammalian and Avian Prion Protein: Structural

Studies Using Synthetic Peptides." Biochemical and Biophysical Research Communications **214**(3): 993-999.
Hsiao, K., H. F. Baker, et al. (1989). "Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome." Nature **338**(6213): 342-345.
Huang, F.-P., C. F. Farquhar, et al. (2002). "Migrating intestinal dendritic cells transport PrPSc from the gut." J Gen Virol **83**(1): 267-271.
Huehn, J., K. Siegmund, et al. (2004). "Developmental Stage, Phenotype, and Migration Distinguish Naive- and Effector/Memory-like CD4+ Regulatory T Cells." The Journal of Experimental Medicine **199**(3): 303-313.
Humphrey, J. H., D. Grennan, et al. (1984). "The origin of follicular dendritic cells in the mouse and the mechanism of trapping of immune complexes on them." European Journal of Immunology **14**(9): 859-864.
Hunter, N., J. Hope, et al. (1987). "Linkage of the Scrapie-associated Fibril Protein (PrP) Gene and Sinc Using Congenic Mice and Restriction Fragment Length Polymorphism Analysis." J Gen Virol **68**(10): 2711-2716.
Ierna, M., C. F. Farquhar, et al. (2006). "Resistance of Neonatal Mice to Scrapie Is Associated with Inefficient Infection of the Immature Spleen." J. Virol. **80**(1): 474-482.
Inman, C. F., L. E. N. Rees, et al. (2005). "Validation of computer-assisted, pixel-based analysis of multiple-colour immunofluorescence histology." Journal of Immunological Methods **302**(1-2): 156-167.
Jackson, G. S., I. Murray, et al. (2001). "Location and properties of metal-binding sites on the human prion protein." Proceedings of the National Academy of Sciences of the United States of America **98**(15): 8531-8535.
Jakob, A. M. (1921a). "Uber eigenartige Erkrankunbunden des Zentralnervensystems mit bemerkenswertem anatomischen Befund (spastische Pseudosclerose-Encephalomyelopathy mit disseminierten degenerationsherden)." Zeitschrift fur die gesamte Neurologie und Psychiatrie **64**(147-228).
Jakob, A. M. (1921b). "Uber eine der multiplen Sklerose klinisch nahestehende Erkrankung desZentralnervensystems (spatische Pseudosklerose) mit bemerkenswerten anatomischen Befunde: Mitteilung einesvierten Falles." Medizinische Klinik **17**: 382-86.
Jeffrey, M., L. , A. González, et al. (2006). "Transportation of prion protein across the intestinal mucosa of scrapie-susceptible and scrapie-resistant sheep." The Journal of Pathology **209**(1): 4-14.
Jeffrey, M., S. Martin, et al. (2003). "Cell-associated variants of disease-specific prion protein immunolabelling are found in different sources of sheep transmissible spongiform encephalopathy." J Gen Virol **84**(4): 1033-1046.
Jeffrey, M., S. Martin, et al. (2001). "Onset and Distribution of Tissue PrP Accumulation in Scrapie-affected Suffolk Sheep as Demonstrated by Sequential Necropsies and Tonsillar Biopsies." Journal of Comparative Pathology **125**(1): 48-57.
Jeffrey, M., G. McGovern, et al. (2000). "Sites of prion protein accumulation in scrapie-infected mouse spleen revealed by immuno-electron microscopy." The Journal of Pathology **191**(3): 323-332.
Jeffrey, M. and G. A. H. Wells (1988). "Spongiform Encephalopathy in a Nyala (Tragelaphus angasi)." Veterinary Pathology Online **25**(5): 398-399.
Joiner, S., J. Linehan, et al. (2002). "Irregular presence of abnormal prion protein in appendix in variant Creutzfeldt-Jakob disease." Journal of Neurology, Neurosurgery & Psychiatry **73**(5): 597-598.

Jones, M., D. Wight, et al. (2009). "An Antibody to the Aggregated Synthetic Prion Protein Peptide (PrP106–126) Selectively Recognizes Disease-Associated Prion Protein (PrPSc) from Human Brain Specimens." Brain Pathology **19**(2): 293-302.

Kaeser, P. S., M. A. Klein, et al. (2001). "Efficient lymphoreticular prion propagation requires prp(c) in stromal and hematopoietic cells." J. Virol. **75**: 7097-7106.

Kaneider, N. C., A. Kaser, et al. (2003). "Sphingosine Kinase-Dependent Migration of Immature Dendritic Cells in Response to Neurotoxic Prion Protein Fragment." J. Virol. **77**(9): 5535-5539.

Kapasi, Z. F., G. F. Burton, et al. (1993). "Induction of functional follicular dendritic cell development in severe combined immunodeficiency mice. Influence of B and T cells." The Journal of Immunology **150**(7): 2648-2658.

Kapasi, Z. F., D. Qin, et al. (1998). "Follicular Dendritic Cell (FDC) Precursors in Primary Lymphoid Tissues." J Immunol **160**(3): 1078-1084.

Kimberlin, R. H. and C. A. Walker (1979). "Pathogenesis of mouse scrapie: Dynamics of agent replication in spleen, spinal cord and brain after infection by different routes." Journal of Comparitive Pathology **89**: 551-562.

Kingsbury, D. T., D. A. Smeltzer, et al. (1981). "Evidence for Normal Cell-Mediated Immunity in Scrapie-Infected Mice." Infect. Immun. **32**(3): 1176-1180.

Kirkwood, J. K., G. A. Wells, et al. (1990). "Spongiform encephalopathy in an arabian oryx (Oryx leucoryx) and a greater kudu (Tragelaphus strepsiceros)." Veterinary Record **127**(17): 418-420.

Kitamoto, T., S. Mohri, et al. (1989). "Organ Distribution of Proteinase-resistant Prion Protein in Humans and Mice with Creutzfeldt-Jakob Disease." J Gen Virol **70**(12): 3371-3379.

Klein, M. A., R. Frigg, et al. (1997). "A crucial role for B cells in neuroinvasive scrapie." Nature **390**(6661): 687-690.

Klein, M. A., R. Frigg, et al. (1998). "PrP expression in B lymphocytes is not required for prion neuroinvasion." Nat Med **4**(12): 1429-1433.

Klein, M. A., P. S. Kaeser, et al. (2001). "Complement facilitates early prion pathogenesis." Nat Med **7**(4): 488-492.

Klickstein, L. B., S. F. Barbashov, et al. (1997). "Complement Receptor Type 1 (CR1, CD35) Is a Receptor for C1q." Immunity **7**(3): 345-355.

Konold, T., S. J. Moore, et al. (2008). "Evidence of scrapie transmission via milk." BMC Veterinary Research **4**(1): 14.

Kopf, M., S. Herren, et al. (1998). "Interleukin 6 Influences Germinal Center Development and Antibody Production via a Contribution of C3 Complement Component." J. Exp. Med. **188**(10): 1895-1906.

Korth, C., B. Stierli, et al. (1997). "Prion (PrPSc)-specific epitope defined by a monoclonal antibody." Nature **390**(6655): 74-77.

Kos, C. H. (2004). "Cre/loxP system for generating tissue-specific knockout mouse models." Nutrition Reviews **6.1**: 243-246.

Kosco-Vilbois, M. H. (2003). "Are follicular dendritic cells really good for nothing?" Nature Reviews Immunology **3**: 764-769.

Kosco-Vilbois, M. H., J.-Y. Bonnefoy, et al. (1997). "The physiology of murine germinal center reactions." Immunological Reviews **156**(1): 127-136.

Kosco, M. H., E. Pflugfelder, et al. (1992). "Follicular dendritic cell-dependent adhesion and proliferation of B cells in vitro." J Immunol **148**(8): 2331-2339.

Kovacs, G. G., E. Lindeck-Pozza, et al. (2004). "Creutzfeldt-Jakob disease and inclusion body myositis: Abundant disease-associated prion protein in muscle." Annals of Neurology **55**(1): 121-125.

Kranich, J., N. J. Krautler, et al. (2008). "Follicular dendritic cells control engulfment of apoptotic bodies by secreting Mfge8." J. Exp. Med.: jem.20071019.

Kraus, M., M. B. Alimzhanov, et al. (2004). "Survival of Resting Mature B Lymphocytes Depends on BCR Signaling via the Ig[alpha]/[beta] Heterodimer." Cell **117**(6): 787-800.

Kretzschmar, H. A., G. Honold, et al. (1991). "Prion protein mutation in family first reported by Gerstmann, Sträussler, and Scheinker." The Lancet **337**(8750): 1160.

Kretzschmar, H. A., S. B. Prusiner, et al. (1986). "Scrapie prionproteins are synthesised in neurones." American Journal of Pathology **122**: 1-5.

Kubosaki, A., S. Yusa, et al. (2001). "Distribution of Cellular Isoform of Prion Protein in T Lymphocytes and Bone Marrow, Analyzed by Wild-Type and Prion Protein Gene-Deficient Mice." Biochemical and Biophysical Research Communications **282**(1): 103-107.

Kurschner, C. and J. I. Morgan (1995). "The cellular prion protein (PrP) selectively binds to Bcl-2 in the yeast two-hybrid system." Molecular Brain Research **30**: 165-168.

Kuwahara, C., A. M. Takeuchi, et al. (1999). "Prions prevent neuronal cell-line death." Nature **400**(6741): 225-226.

Ladeby, R., M. Wirenfeldt, et al. (2005). "Microglial cell population dynamics in the injured adult central nervous system." Brain Research Reviews **48**(2): 196-206.

Lasmézas, C. I. (2003). "Putative functions of PrP^{c}." British Medical Bulletin **66**: 61-70.

Lasmézas, C. I., J.-P. Deslys, et al. (1997). "Transmission of the BSE Agent to Mice in the Absence of Detectable Abnormal Prion Protein." Science **275**(5298): 402-404.

Legname, G., I. V. Baskakov, et al. (2004). "Synthetic Mammalian Prions." Science **305**(5684): 673-676.

Lemzi, S., F. Ronzon, et al. (2006). "PrPd accumulation in organs of ARQ/ARQ sheep experimentally infected with BSE by peripheral routes." Acta Biochimica Polonica **53**(1): 399-405.

Leopoldt, J. G. (1750). Nützliche und auf die Erfahrung gegründete Einleitung zu der Landwirthschafft. Sorau, Johann Gottlieb Rothen.

Li, R., D. Liu, et al. (2001). "The Expression and Potential Function of Cellular Prion Protein in Human Lymphocytes." Cellular Immunology **207**(1): 49-58.

Liberski, P. P., B. Sikorska, et al. (2008). "Tubulovesicular structures are a consistent (and unexplained) finding in the brains of humans with prion diseases." Virus Research **132**(1-2): 226-228.

Liedtke, W., W. Edelmann, et al. (1996). "GFAP Is Necessary for the Integrity of CNS White Matter Architecture and Long-Term Maintenance of Myelination." Neuron **17**(4): 607-615.

Ligios, C., G. M. Cancedda, et al. (2007). "Intraepithelial and Interstitial Deposition of Pathological Prion Protein in Kidneys of Scrapie-Affected Sheep." PLoS ONE **2**(9): e859.

Ligios, C., C. J. Sigurdson, et al. (2005). "PrPSc in mammary glands of sheep affected by scrapie and mastitis." Nat Med **11**(11): 1137-1138.

Linden, R., V. R. Martins, et al. (2008). "Physiology of the Prion Protein." Physiological Reviews **88**(2): 673-728.
Liu, T., R. Li, et al. (2001). "Normal Cellular Prior Protein Is Preferentially Expressed on Subpopulations of Murine Hemopoietic Cells." The Journal of Immunology **166**(6): 3733-3742.
Liu, Y.-J., J. Xu, et al. (1997). "Follicular Dendritic Cells Specifically Express the Long CR2/CD21 Isoform." J. Exp. Med. **185**(1): 165-170.
Llewelyn, C. A., P. E. Hewitt, et al. (2004). "Possible transmission of variant Creutzfeldt-Jacob disease by blood transfusion." The Lancet **363**(9407): 417-721.
Llewelyn, C. A., P. E. Hewitt, et al. (2004). "Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion." The Lancet **363**(9407): 417-421.
Loeuillet, C., C. Lemaire-Vielle, et al. (2010). "Prion replication in the haematopoietic compartment is not required for neuroinvasion in scrapie mouse model." PLoS ONE **5**(10): 1-6.
Mabbot, N., McGovern, G, Jeffrey, M and Bruce, ME (2002). "Temporary blockade of the tumour necrosis factor receptor signalling pathway impedes the spread of scrapie to the brain." Journal of Virology **76**(10): 5131-5139.
Mabbot, N. A., Bruce, M.E., Botto, M., Walport, M.J. and Pepys, M.B. (2001). "Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie." Nature Medicine **7**: 485-487.
Mabbott, N. A. (2004). "The complement system in prion diseases." Current Opinion in Immunology **16**(5): 587-593.
Mabbott, N. A., K. L. Brown, et al. (1997). "T-lymphocyte activation and the cellular form of the prion protein." Immunology **92**(2): 161-165.
Mabbott, N. A. and M. E. Bruce (2002). "Follicular dendritic cells as targets for intervention in transmissible spongiform encephalopathies." Seminars in Immunology **14**(4): 285-293.
Mabbott, N. A., M. E. Bruce, et al. (2001). "Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie." Nat Med **7**(4): 485-487.
Mabbott, N. A., F. Mackay, et al. (2000b). "Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie." Nat Med **6**(7): 719-720.
Mabbott, N. A., G. McGovern, et al. (2002). "Temporary Blockade of the Tumor Necrosis Factor Receptor Signaling Pathway Impedes the Spread of Scrapie to the Brain." J. Virol. **76**(10): 5131-5139.
Mabbott, N. A., A. Williams, et al. (2000a). "Tumor Necrosis Factor Alpha-Deficient, but Not Interleukin-6-Deficient, Mice Resist Peripheral Infection with Scrapie." J. Virol. **74**(7): 3338-3344.
Mabbott, N. A., J. Young, et al. (2003). "Follicular Dendritic Cell Dedifferentiation by Treatment with an Inhibitor of the Lymphotoxin Pathway Dramatically Reduces Scrapie Susceptibility." J. Virol. **77**(12): 6845-6854.
Mackay, F. and J. L. Browning (1998). "Turning off follicular dendritic cells." Nature **395**(6697): 26-27.
Maeda, K., M. Matsuda, et al. (1995). "Follicular dendritic cells: Structure as related to function." Current Topics in Microbiology and Immunology **201**: 119-139.
Mallucci, G. R., S. Ratte, et al. (2002). "Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration." EMBO J **21**(3): 202-210.

Manson, J. C., A. R. Clarke, et al. (1994a). "129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal." Molecular Neurobiology **8**: 121-127.

Manson, J. C., A. R. Clarke, et al. (1994b). "PrP gene dosage determines the timing but not the final intensity or distribution of lesions in scrapie pathology." Neurodegeneration **3**: 331-340.

Manuelidis, L. (2007). "A 25nm virion is the likely cause of transmissible spongiform encephalopathies." Journal of Cellular Biochemistry **100**(4): 897-915.

Mao, X., Y. Fujiwara, et al. (1999). "Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice." PNAS **96**(9): 5037-5042.

Marsh, R. F., I. C. Pan, et al. (1970). "Failure to Demonstrate Specific Antibody in Transmissible Mink Encephalopathy." Infect. Immun. **2**(6): 727-730.

McBride, P. A., P. Eikelenboom, et al. (1992). "PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice." The Journal of Pathology **168**(4): 413-418.

McGovern, G., K. L. Brown, et al. (2004). "Murine Scrapie Infection Causes an Abnormal Germinal Centre Reaction in the Spleen." Journal of Comparative Pathology **130**(2-3): 181-194.

McGovern, G. and M. Jeffrey (2007). "Scrapie-Specific Pathology of Sheep Lymphoid Tissues." PLoS ONE **2**(12): e1304.

McGovern, G., N. A. Mabbott, et al. (2009a). "Scrapie Affects the Maturation Cycle and Immune Complex Trapping by Follicular Dendritic Cells in Mice." PLoS ONE **4**(12): e8186.

McKinley, M. P., D. C. Bolton, et al. (1983). "A protease-resistant protein is a structural component of the scrapie prion." Cell **35**: 57-62.

Mebius, R. E., S. van Tuijl, et al. (1998). "Transfer of Primitive Stem/Progenitor Bone Marrow Cells from LT{alpha}-/- Donors to Wild-Type Hosts: Implications for the Generation of Architectural Events in Lymphoid B Cell Domains." J Immunol **161**(8): 3836-3843.

Medori, R., H.-J. Tritschler, et al. (1992). "Fatal Familial Insomnia, a Prion Disease with a Mutation at Codon 178 of the Prion Protein Gene." New England Journal of Medicine **326**(7): 444-449.

Meyer, R. K., M. P. McKinley, et al. (1986). "Separation and Properties of Cellular and Scrapie Prion Proteins." PNAS **83**(8): 2310-2314.

Mikol, J. (1999). "Neuropathology of prion diseases." Biomedicine and pharmacotherapy **53**: 19-26.

Miller, J. J. and L. J. Cole (1967). "The Radiation Resistance of Long-Lived Lymphocytes and Plasma Cells in Mouse and Rat Lymph Nodes." J Immunol **98**(5): 982-990.

Mitchell, D. A., L. Kirby, et al. (2007). "Prion protein activates and fixes complement directly via the classical pathway: Implications for the mechanism of scrapie agent propagation in lymphoid tissue." Molecular Immunology **44**(11): 2997-3004.

Mitteregger, G., M. Vosko, et al. (2007). "The Role of the Octarepeat Region in Neuroprotective Function of the Cellular Prion Protein." Brain Pathology **17**(2): 174-183.

Mohan, J., K. L. Brown, et al. (2004). "Scrapie transmission following exposure through the skin is dependent on follicular dendritic cells in lymphoid tissues." Journal of Dermatological Science **35**(2): 101-111.

Mohan, J., M. E. Bruce, et al. (2005). "Follicular dendritic cell dedifferentiation reduces scrapie susceptibility following inoculation via the skin." Immunology **114**(2): 225-234.

Montrasio, F. (2000). "Impaired prion replication in spleens of mice lacking functional follicular dendritic cells." Science **288**: 1257-1259.

Montrasio, F., A. Cozzio, et al. (2001). "B lymphocyte-restricted expression of prion protein does not enable prion replication in prion protein knockout mice." PNAS **98**(7): 4034-4037.

Morgan, B. P., K. J. Marchbank, et al. (2005). "Complement: central to innate immunity and bridging to adaptive responses." Immunology Letters **97**(2): 171-179.

Moroncini, G., N. Kanu, et al. (2004). "Motif-grafted antibodies containing the replicative interface of cellular PrP are specific for PrPSc." Proceedings of the National Academy of Sciences of the United States of America **101**(28): 10404-10409.

Mouillet-Richard, S., M. Ermonval, et al. (2000). "Signal Transduction Through Prion Protein." Science **289**(5486): 1925-1928.

Munoz-Fernandez, R., F. J. Blanco, et al. (2006). "Follicular Dendritic Cells Are Related to Bone Marrow Stromal Cell Progenitors and to Myofibroblasts." J Immunol **177**(1): 280-289.

Murakami, T., X. Chen, et al. (2007). "Splenic CD19 CD35+B220+ cells function as an inducer of follicular dendritic cell network formation." Blood **110**(4): 1215-1224.

Nico, P. B. C., F. de-Paris, et al. (2005). "Altered behavioural response to acute stress in mice lacking cellular prion protein." Behavioural Brain Research **162**(2): 173-181.

Nielsen, C. H., Fischer, E.M. and Leslie, R.G.Q. (2000). "The role of complement in the acquired immune response." Immunology **100**: 4-12.

Oesch, B., D. Westaway, et al. (1985). "A cellular gene encodes scrapie PrP 27-30 protein." Cell **40**: 735-746.

Ohsawa, K., Y. Imai, et al. (2004). "Microglia/macrophage-specific protein Iba1 binds to fimbrin and enhances its actin-bundling activity." Journal of Neurochemistry **88**(4): 844-856.

Oldstone, M. B. A., R. Race, et al. (2002). "Lymphotoxin-{alpha}- and Lymphotoxin-{beta}-Deficient Mice Differ in Susceptibility to Scrapie: Evidence against Dendritic Cell Involvement in Neuroinvasion." J. Virol. **76**(9): 4357-4363.

Paitel, E., C. Alves da Costa, et al. (2002). "Overexpression of PrPc triggers caspase 3 activation: potentiation by proteasome inhibitors and blockade by anti-PrP antibodies." Journal of Neurochemistry **83**(5): 1208-1214.

Pan, K., M. Baldwin, et al. (1993). "Conversion of {alpha}-Helices {beta}-Sheets Features in the Formation of the scrapie Prion Proteins." PNAS **90**(23): 10962-10966.

Paramithiotis, E., M. Pinard, et al. (2003). "A prion protein epitope selective for the pathologically misfolded conformation." Nature Medicine **9**(7): 893-899.

Parchi, P., R. Castellani, et al. (1995). "Protease-Resistant Prion Protein in Sporadic Creutzfeldt-Jakob Disease (Cjd): Correlation With Clinico-Pathological Features and Prp Genotype: 19." Journal of Neuropathology & Experimental Neurology **54**(3): 416.

Pasparakis, M., L. Alexopoulou, et al. (1996). "Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the

formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response." J. Exp. Med. **184**(4): 1397-1411.
Peden, A. H., M. W. Head, et al. (2004). "Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient." The Lancet **364**(9433): 527-529.
Peden, A. H., L. McCardle, et al. (2010). "Variant CJD infection in the spleen of a neurologically asymptomatic UK adult patient with haemophilia."
Petsch, B., A. Muller-Schiffmann, et al. (2011). "Biological effects and use of PrPSc- and PrP-specific antibodies generated by immunizing with purified full length native mouse prions." J. Virol.: JVI.02467-10.
Phan, T. G., I. Grigorova, et al. (2007). "Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells." Nat Immunol **8**(9): 992-1000.
Piccardo, P., J. C. Manson, et al. (2007). "Accumulation of prion protein in the brain that is not associated with transmissible disease." PNAS **104**(11): 4712-4717.
Porter, D. D., H. G. Porter, et al. (1973). "Failure to Demonstrate a Humoral Immune Response to Scrapie Infection in Mice." The Journal of Immunology **111**(5): 1407-1410.
Prinz, M., M. Heikenwalder, et al. (2003). "Positioning of follicular dendritic cells within the spleen controls prion neuroinvasion." Nature **425**(6961): 957-962.
Prinz, M., F. Montrasio, et al. (2002). "Lymph nodal prion replication and neuroinvasion in mice devoid of follicular dendritic cells." PNAS **99**(2): 919-924.
Prodeus, A. P., S. Goerg, et al. (1998). "A Critical Role for Complement in Maintenance of Self-Tolerance." Immunity **9**(5): 721-731.
Prusiner, S. (1982). "Novel proteinaceous infectious particles cause scrapie." Science **216**(4542): 136-144.
Prusiner, S. B. (1982). "Novel proteinaceous infectious particles cause scrapie." Science **216**(4542): 136-144.
Prusiner, S. B. (1991). "Molecular biology of prion diseases." Science **252**(5012): 1515-1522.
Prusiner, S. B., M. R. Scott, et al. (1998). "Prion Protein Biology." Cell **93**(3): 337-348.
Qin, D., J. Wu, et al. (2000). "Fcγ Receptor IIB on Follicular Dendritic Cells Regulates the B Cell Recall Response." The Journal of Immunology **164**(12): 6268-6275.
Raymond, C. R., P. Aucouturier, et al. (2007). "In Vivo Depletion of CD11c+ Cells Impairs Scrapie Agent Neuroinvasion from the Intestine." J Immunol **179**(11): 7758-7766.
Raymond, C. R. and N. A. Mabbott (2007). "Assessing the involvement of migratory dendritic cells in the transfer of the scrapie agent from the immune to peripheral nervous systems." Journal of Neuroimmunology **187**(1-2): 114-125.
regulation, E. C. (2001). Rules for prevention, control and eradication of certain transmissible spongiform encephalopathies. E. Commission. **Regulation, (EC) No 99/2001 of the European Parliament and of the Council of 22 May 2001**.
regulation, E. C. (2004). Amendment to regulation (EC) No 999/2001 of the European Parliament and of the Council regarding eradication methods for transmissible spongiform encephalopathies in bovine, ovine and caprine animals, the trade and importation of semen and embryos of ovine and caprine animals and

specified risk material. E. Commission. **Commission regulation (EC) No 1492/2004 of 23 August 2004**.

regulation, E. C. (2007). Amendment to annex VII to Regulation (EC) No 999/2001 of the European Parliament and of the Council regarding rules for prevention, control and eradication of certain transmissible spongiform encephalopathies. E. Commission. **Commission Regulation (EC) No 1428/2007 of 4 December 2007**.

Reynes, M., J. P. Aubert, et al. (1985). "Human follicular dendritic cells express CR1, CR2, and CR3 complement receptor antigens." J Immunol **135**(4): 2687-2694.

Ritchie, D. L., K. L. Brown, et al. (1999). "Visualisation of PrP protein and follicular dendritic cells in uninfected and scrapie infected spleen." Journal of Cellular Pathology **1**(3-10).

Rogers, M., F. Yehiely, et al. (1993). "Conversion of Truncated and Elongated Prion Proteins into the Scrapie Isoform in Cultured Cells." PNAS **90**(8): 3182-3186.

Ryder, S., G. Dexter, et al. (2009). "Accumulation and dissemination of prion protein in experimental sheep scrapie in the natural host." BMC Veterinary Research **5**(1): 9.

Sakaguchi, S., S. Katamine, et al. (1995). "Accumulation of proteinase K-resistant prion protein (PrP) is restricted by the expression level of normal PrP in mice inoculated with a mouse-adapted strain of the Creutzfeldt-Jakob disease agent." J. Virol. **69**(12): 7586-7592.

Sandberg, M. K., H. Al-Doujaily, et al. (2011). "Prion propagation and toxicity in vivo occur in two distinct mechanistic phases." Nature **470**(7335): 540-542.

Sarnataro, D., V. Campana, et al. (2004). "PrP^{C} Association with Lipid Rafts in the Early Secretory Pathway Stabilizes Its Cellular Conformation." Mol. Biol. Cell **15**(9): 4031-4042.

Schiffer, D., M. T. Giordana, et al. (1986). "Glial fibrillary acidic protein and vimentin in the experimental glial reaction of the rat brain." Brain Research **374**(1): 110-118.

Schmidt-Supprian, M. and K. Rajewsky (2007). "Vagaries of conditional gene targeting." Nat Immunol **8**(7): 665-668.

Schneider, K., H. Fangerau, et al. (2008a). "The early history of the transmissible spongiform encephalopathies exemplified by scrapie." Brain Research Bulletin **77**(6): 343-355.

Schulz-Schaeffer, W. J., S. Tschoke, et al. (2000). "The paraffin-embedded tissue blot detects PrP^{Sc} early in the incubation time in prion diseases." American Journal of Pathology **156**: 51-56.

Schwaeble, W., M. K. Schafer, et al. (1995). "Follicular dendritic cells, interdigitating cells, and cells of the monocyte-macrophage lineage are the C1q-producing sources in the spleen. Identification of specific cell types by in situ hybridization and immunohistochemical analysis." J Immunol **155**(10): 4971-4978.

Seifert, G., K. Schilling, et al. (2006). "Astrocyte dysfunction in neurological disorders: a molecular perspective." Nat Rev Neurosci **7**(3): 194-206.

Shiraishi, N., Utsunomiya, H, Nishikimi, M (2006). "Combination of NADPH and copper ions generates proteinase K-resistant aggregates for recombinant prion protein." Journal of Biological Chemistry **281**(46): 34880-34887.

Sigurdson, C. J., E. S. Williams, et al. (1999). "Oral transmission and early lymphoid tropism of chronic wasting disease PrPres in mule deer fawns (Odocoileus hemionus)." J Gen Virol **80**(10): 2757-2764.

Sixbey, J. W., S. M. Lemon, et al. (1986). "A second site for Epstein-Barr virus shedding: The uterine cervix." The Lancet **328**(8516): 1122-1124.
Somerville, R. A. (1999). "Host and transmissible spongiform encephalopathy agent strain control glycosylation of PrP." J Gen Virol **80**(7): 1865-1872.
Somerville, R. A., C. R. Birkett, et al. (1997). "Immunodetection of PrPSc in spleens of some scrapie-infected sheep but not BSE-infected cows." J Gen Virol **78**(9): 2389-2396.
Somerville, R. A., A. Chong, et al. (1997). "Biochemical typing of scrapie strains." Nature **386**: 564.
Somerville, R. A., P. A. Merz, et al. (1986). "Partial Copurification of Scrapie-Associated Fibrils and Scrapie Infectivity." Intervirology **25**(1): 48-55.
Spielhaupter, C. and H. M. Schätzl (2001). "PrP^C Directly Interacts with Proteins Involved in Signaling Pathways." Journal of Biological Chemistry **276**(48): 44604-44612.
Stahl, N., D. R. Borchelt, et al. (1987). "Scrapie prion protein contains a phosphatidylinositol glycolipid." Cell **51**(2): 229-240.
Stimson, E., J. Hope, et al. (1999). "Site-Specific Characterization of the N-Linked Glycans of Murine Prion Protein by High-Performance Liquid Chromatography/Electrospray Mass Spectrometry and Exoglycosidase Digestions†." Biochemistry **38**(15): 4885-4895.
Stoorvogel, W., M. J. Kleijmeer, et al. (2002). "The Biogenesis and Functions of Exosomes." Traffic **3**(5): 321-330.
Swartzendruber, W. F. and C. C. Congdon (1963). "Electron microscopic observations on tingible body macrophages in mouse spleen." Journal of Cell Biology **19**: 641-46.
Szakal, A. K. and J. G. Tew (1992). "Follicular Dendritic Cells: B-Cell Proliferation and Maturation." Cancer Res **52**(19_Supplement): 5554s-5556.
Takahashi, K., Y. Kozono, et al. (1997). "Mouse complement receptors type 1 (CR1;CD35) and type 2 (CR2;CD21): expression on normal B cell subpopulations and decreased levels during the development of autoimmunity in MRL/lpr mice." J Immunol **159**(3): 1557-1569.
Taylor, D. M. (1993). "Inactivation of TSE agents." British Medical Bulletin **49**(4): 810-821.
Taylor, D. M., K. Fernie, et al. (1998). "Observations on thermostable subpopulations of the unconventional agents that cause transmissible degenerative encephalopathies." Veterinary Microbiology **64**(1): 33-38.
Terry, L. A., S. Marsh, et al. (2003). "Detection of disease-specific PrP in the distal ileum of cattle exposed orally to the agent of bovine spongiform encephalopathy." Vet Rec. **152**(13): 387-392.
Tew, J. G., M. H. Kosco, et al. (1989). "The alternative antigen pathway." Immunology Today **10**(7): 229-232.
Tew, J. G. and T. E. Mandel (1979). "Prolonged antigen half-life in the lymphoid follicles of specifically immunized mice."
Thaer, S. (1821). Läßt sich irgend ein Grund der ersten Entstehung der Traber-oder Kreutzdreher- Krankheit annehmen?
Thery, C., A. Regnault, et al. (1999). "Molecular Characterization of Dendritic Cell-derived Exosomes: Selective Accumulation of the Heat Shock Protein hsc73." J. Cell Biol. **147**(3): 599-610.
Thielen, C., N. Antoine, et al. (2001). "Human FDCs express PrP^C *in vivo* and *in vitro*." Developmental Immunology **8**: 259-66.

Tobler, I., S. E. Gaus, et al. (1996). "Altered circadian activity rhythms and sleep in mice devoid of prion protein." Nature **380**(6575): 639-642.
Traggiai, E., L. Chicha, et al. (2004). "Development of a Human Adaptive Immune System in Cord Blood Cell-Transplanted Mice." Science **304**(5667): 104-107.
Tsoukas, C. D. and J. D. Lambris (1988). "Expression of CR2/EBV receptors on human thymocytes detected by monoclonal antibodies." European Journal of Immunology **18**(8): 1299-1302.
Tuzi, N. L., A. R. Clarke, et al. (2004). "Cre-loxP mediated control of PrP to study transmissible spongiform encephalopathy diseases." genesis **40**(1): 1-6.
van der Berg, T. K., K. Yoshida, et al. (1995). "Mechanisms of immune complex trapping by follicular dendritic cells." Current Topics in Microbiology and Immunology **201**: 49-63.
Van Everbroeck, B., P. Pals, et al. (2002). "Transmissible spongiform encephalopathies: the story of a pathogenic protein." Peptides **23**(7): 1351-1359.
van Keulen, L. J., B. E. Schreuder, et al. (1996). "Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie." J. Clin. Microbiol. **34**(5): 1228-1231.
Victoratos, P., J. Lagnel, et al. (2006). "FDC-Specific Functions of p55TNFR and IKK2 in the Development of FDC Networks and of Antibody Responses." Immunity **24**(1): 65-77.
Vidal, E., R. Tortosa, et al. (2008). "Lack of PrPsc immunostaining in intracranial ectopic lymphoid follicles in a sheep with concomitant non-suppurative encephalitis and Nor98-like atypical scrapie: A case report." The Veterinary Journal **177**(2): 283-288.
Vidal, E., R. Tortosa, et al. (2008). "Lack of PrPsc immunostaining in intracranial ectopic lymphoid follicles in a sheep with concomitant non-suppurative encephalitis and Nor98-like atypical scrapie: A case report." The Veterinary Journal **177**(2): 283-288.
Videm, V. and M. Albrigtsen (2008). "Soluble ICAM-1 and VCAM-1 as Markers of Endothelial Activation." Scandinavian Journal of Immunology **67**(5): 523-531.
Voigt, I. (2000). "CXCR5-deficient mice develop functional germinal centers in the splenic T cell zone." Eur. J. Immunol. **30**: 560-567.
von Poser-Klein, C., E. Flechsig, et al. (2008). "Alteration of B-Cell Subsets Enhances Neuroinvasion in Mouse Scrapie Infection." J. Virol. **82**(7): 3791-3795.
von Richthofen, A. K. S. F. (1827). Die Traberkrankheit der Scaafe, verglichen mit der sogenannten Schaafräudekrankheit. Breslau, Wilhelm Gottlieb Korn.
Vorberg, I., A. Raines, et al. (2004). "Susceptibility of Common Fibroblast Cell Lines to Transmissible Spongiform Encephalopathy Agents." The Journal of Infectious Diseases **189**(3): 431-439.
Wagner, C. and G. M. Hansch (2006). "Receptors for complement C3 on T-lymphocytes: Relics of evolution or functional molecules?" Molecular Immunology **43**(1-2): 22-30.
Walz, R., O. B. Amaral, et al. (1999). "Increased Sensitivity to Seizures in Mice Lacking Cellular Prion Protein." Epilepsia **40**(12): 1679-1682.
Wang, G., X. M. Zhou, et al. (2010). "Cellular prion protein released on exosomes from macrophages binds to Hsp70." Acta Biochim Biophys Sin **42**(5): 345-350.

Wells, G. A., A. C. Scott, et al. (1987). "A novel progressive spongiform encephalopathy in cattle." Veterinary Record **121**: 419-20.
Wen, L., S. A. Shinton, et al. (2005). "Association of B-1 B Cells with Follicular Dendritic Cells in Spleen." J Immunol **174**(11): 6918-6926.
Westaway, D., S. J. DeArmond, et al. (1994). "Degeneration of skeletal muscle, peripheral nerves, and the central nervous system in transgenic mice overexpressing wild-type prion proteins." Cell **76**(1): 117-129.
Westaway, D. and S. B. Prusiner (1986). "Conservation of the cellular gene encoding the scrapie prion protein." Nucleic Acids Research **14**(5): 2035-2044.
Wilesmith, J. W., J. B. M. Ryan, et al. (1991). "Bovine spongiform encephalopathy: epidemiological studies on the origin." Veterinary Record **128**(9): 199-203.
Will, R. G., J. W. Ironside, et al. (1996). "A new variant of Creutzfeldt-Jakob disease in the UK." The Lancet **347**(9006): 921-925.
Wroe, S. J., S. Pal, et al. (2006). "Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report." The Lancet **368**(9552): 2061-2067.
Wu, X., N. Jiang, et al. (2000). "Impaired Affinity Maturation in Cr2-/- Mice Is Rescued by Adjuvants Without Improvement in Germinal Center Development." The Journal of Immunology **165**(6): 3119-3127.
Wyatt, J. M., G. R. Pearson, et al. (1991). "Naturally occurring scrapie-like spongiform encephalopathy in five domestic cats." Veterinary Record **129**(11): 233-236.
Zabel, M. D., M. Heikenwalder, et al. (2007). "Stromal Complement Receptor CD21/35 Facilitates Lymphoid Prion Colonization and Pathogenesis." J Immunol **179**(9): 6144-6152.
Zanusso, G., S. Ferrari, et al. (2003). "Detection of Pathologic Prion Protein in the Olfactory Epithelium in Sporadic Creutzfeldt–Jakob Disease." New England Journal of Medicine **348**(8): 711-719.
Zhang, C. C., A. D. Steele, et al. (2006). "Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal." Proceedings of the National Academy of Sciences of the United States of America **103**(7): 2184-2189.
Zhou, X. M., G. X. Xu, et al. (2008). "In vitro effect of prion peptide PrP 106-126 on mouse macrophages: Possible role of macrophages in transport and proliferation for prion protein." Microbial Pathogenesis **44**(2): 129-134.
Zigas, V. and D. C. Gadusek (1957). "Kuru: clinical study of a new syndrome resembling paralysis agitans in the natives of the Eastern Highlands of Australian New Guinea " Medical Journal of Australia **2**: 245-54.
Zlotnik, I. and J. C. Rennie (1963). "Further observations on the experimental transmission of scrapie from sheep and goats to experimental mice." Jounal of Comparitive Pathology **73**: 150-162.
Zlotnik, I. and J. C. Rennie (1965). "Experimental transmission of mouse passaged scrapie to goats, sheep, rats and hamsters." Journal of Comparative Pathology **75**(2): 147-156.

Appendix I

ImageJ macro- Multiple colour backgrounds

This macro was used to determine background levels of immunolabelling in fluorescently labelled images and to determine threshold levels to use in further image analysis. A line is drawn through the image using ImageJ software, ensuring that line passes through areas of positive bright immunolabelling, areas of positive lower level immunolabelling and background levels of colour present. The intensity of colour for all plots along that line is then plotted out on a graph. This graph allows the determination of a threshold of colour intensity to eliminate background staining from further analysis of images.

```
// Generic multiple colour macro for assisting setting of thresholds
// Analyses pixel intensities along a line selection
// for a series of greyscale slices in a stack, each corresponding to a colour channel
// Expandable to any number of colour channels (slices)

// Check for RGB image and convert to greyscale stack
// Breaks if image name includes "(RGB)"
image = getImageID(); selectImage(image); info = getInfo();
if (indexOf(info, "(RGB)") >1) {
        getLine(x1,y1,x2,y2,width);
        run("RGB Split");
        run("Convert Images to Stack");
        makeLine(x1,y1,x2,y2);}

Greyscales = 255; if (indexOf(info, "pixel: 16")>1) {Greyscales = 6553)

setLineWidth(1);
getLine(startx, starty, endx, endy, temp);
stack = getImageID();

selectImage(stack); slices = nSlices();


for (i =0; i<slices; i++) {
        selectImage(stack);

slice = "slice="+(i+1);
        run("Set Slice...", slice);
        ydata = getProfile();
        Plot.create(slice, "pixels", "intensity");
        Plot.setLimits(0, ydata.length, 0, Greyscales);
        Plot.setLineWidth(1);
        Plot.setColor("red");
        Plot.add("line", ydata);
```

Appendix II

ImageJ macro- Multiple colour analysis

This macro was used to measure co-localisation of proteins in immunolabelled fluorescent images. Background levels on immunolabelling were excluded from measurements using the multiple colour backgrounds macro (Appendix I) to set a threshold for positive labelling. Each pixel in the image is counted and recorded as black (no labelling), red, green, blue, yellow (red and green together), magenta (red and blue together), cyan (green and blue together) or white (red, green and blue). Quantification of area of image single or double immunolabelled for each protein can then be used to perform statistical analysis of co-localisation present.

```
// Generic multiple colour co-localisation analysis macro
// Analyses a series of greyscale slices in a stack, each corresponding to a colour channel
// Expandable to any number of colour channels (slices)
// Provides number of pixels above threshold for each combination of colour channels
// Data provided is in bins corresponding to (for three channels):
//
// Bin  Ch 1  Ch 2  Ch 3
// 0    -     -     -
// 1    +     -     -
// 2    -     +     -
// 3    +     +     -
// 4    -     -     +
// 5    +     -     +
// 6    -     +     +
// 7    +     +     +

// Set threshold values for each colour channel (each slice)
// Add extra thresholds for more than 5 channels
Threshold = newArray(6);

Threshold[1] = 10;
Threshold[2] = 40;
Threshold[3] = 50;
Threshold[4] = 50
Threshold[5] = 50;
// Check for RGB image and convert to greyscale stack
// Where Ch 1 = red, Ch 2 = green, Ch 3 = blue
// Breaks if image name includes "(RGB)"
image = getImageID(); selectImage(image); info = getInfo();
if (indexOf(info, "(RGB)") >1) {
        run("RGB Split");
        run("Convert Images to Stack");
```

```
        exit("Draw your ROI and rerun the macro");}

stack = getImageID(); selectImage(stack); slices = nSlices();
results = newArray(pow(2, slices));
Greyscales = 255; if (indexOf(info, "pixel: 16")>1) {Greyscales = 65535;}
//run("Measure");


setBackgroundColor(0,0,0);
for (i =0; i<slices; i++) {
        selectImage(stack);
        slice = "slice="+(i+1);
        run("Set Slice...", slice); run("Clear Outside", "slice");
        changeValues(0,Threshold[i+1],0);
changeValues((Threshold[i+1]+1),Greyscales,pow(2,i));}

run("Z Project...", "start=1 stop=5 projection='Sum Slices'"); sum = getImageID();
selectImage(stack);
selectImage(sum);
run("Restore Selection");

run("Histogram", "bins=" + pow(2,slices) + "  x_min=0 x_max=" + pow(2,slices));
```